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Review Article

CHEMISTRY AND BIOCHEMISTRY OF 4-HYDROXYNONENAL, MALONALDEHYDE AND RELATED ALDEHYDES

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Abstract—Lipid peroxidation often occurs in response to oxidative stress, and a great diversity of aldehydes are formed when lipid hydroperoxides break down in biological systems. Some of these aldehydes are highly reactive and may be considered as second toxic messengers which disseminate and augment initial free radical events. The aldehydes most intensively studied so far are 4-hydroxynonenal, 4-hydroxyhexenal, and malonaldehyde. The purpose of this review is to provide a comprehensive summary on the chemical properties of these aldehydes, the mechanisms of their formation and their occurrence in biological systems and methods for their determination. We will also review the reactions of 4-hydroxyalkenals and malonaldehyde with biomolecules (amino acids, proteins, nucleic acid bases), their metabolism in isolated cells and excretion in whole animals, as well as the many types of biological activities described so far, including cytotoxicity, genotoxicity, chemotactic activity, and effects on cell proliferation and gene expression. Structurally related compounds, such as acrolein, crotonaldehyde, and other 2-alkenals are also briefly discussed, since they have some properties in common with 4-hydroxyalkenals.

Keywords—4-Hydroxynonenal, 4-Hydroxyhexenal, Malonaldehyde, Acrolein, Lipid peroxidation, Free radicals, Biological effects

INTRODUCTION

There is increasing, though not conclusive evidence that aldehydes generated endogenously during the process of lipid peroxidation are causally involved in some of the pathophysiological effects associated with oxidative stress in cells and tissues. Unlike reactive free radicals, aldehydes are rather long lived and can therefore diffuse from the side of their origin (i.e., membranes) and reach and attack targets intracellularly or extracellularly which are distant from the initial free radical event (Fig. 1). Lipid peroxidation on its own is an amplifier for the initial free radicals and the reactive aldehydes generated in this process may well act as "second toxic messengers" of the complex chain reactions which are initiated if polyunsaturated fatty acids of the membrane bilayer are converted to lipid hydroperoxides. The early literature on biological effects of aldehydes was reviewed in 1977.[1] Later, several articles were published, which dealt with some selected and specific aspects of aldehydic lipid peroxidation products. The literature on the occurrence and formation of aldehydes was recently extensively reviewed[2–6] as were the methods for their determination.[7,8] Several reviews also exist which discuss the biological properties of 4-hydroxyalkenals and related α,β-unsaturated aldehydes.[3,9–14] When we were invited to write this review, we first hesitated to add another article to this already long list. What we and likely other colleagues missed, was a review dealing also with the chemical aspects of aldehydic lipid peroxidation products, for example, mechanism of reactions with biomolecules, structures of defined reaction products, or homologies to structurally related aldehydes. A large body of this new review therefore deals with the chemistry of aldehydes as related to their biochemical and biological properties.

Among the many different aldehydes which can be formed during lipid peroxidation, most intensively studied were malonaldehyde (MDA) and 4-hydroxyalkenals, in particular 4-hydroxynonenal (HNE) and

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We dedicate this article to Prof. Dr. T.F. Slater on the occasion of his 60th birthday.

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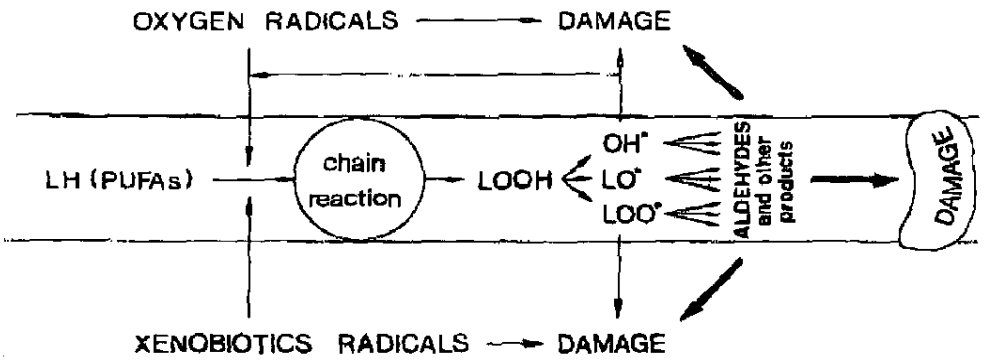

Fig. 1. Scheme for possible routes leading to free radical damage. Free radicals can directly attack critical target molecules or attack polyunsaturated fatty acids (PUFAS) in membranes and inititate lipid peroxidation. This acts as an amplifier, more radicals are formed, and PUFAS are degraded to a variety of products. Some of them as aldehydes are very reactive and have the potential to damage molecules within or outside the membrane.

4-hydroxyhexenal. We try to present a balanced compen- dium on the chemistry, biochemistry, and biology of MDA and 4-hydroxy alkenals. Some structurally related aldehydes, such as acrolein or crotonal, will also briefly be discussed since they have some properties in common with 4-hydroxyalkenals or MDA.

4-HYDROXYALKENALS

History

4-Hydroxyalkenals were discovered in the early 1960s in studies which aimed at the characterization of carcinostatic and cytotoxic substances present in autoxidized methyl linoleate.[15,16] The occurrence of such aldehydes in autoxidized polyunsaturated fatty acids and triglycerides was later confirmed independently by several groups. In 1977 the group of Nakamura[17] reported that 4-hydroxyhexenal is present in autoxidized methyl linolenate, and in 1982[18] the same group found HNE, 4-hydroxyhexenal, 4-hydroperoxynonenal, and 4,5-dihydroxyheptenal in autoxidized linseed oil. Esterbauer et al. reported that HNE is formed from linoleic acid and arachidonic acid[2] and 15-hydroperoxy arachidonic acid[19] in aqueous solution supplemented with ascorbate (0.5 mM) and $FeSO_4$ (0.02 mM). Oarada et al.[20] have identified HNE as one of the major toxic substances in the low molecular weight fraction of methyl linoleate, which was allowed to oxidize in the dark at 12 °C for 72 h to a peroxide value of 1000 meq/kg. Kaneko et al. reported in 1987[21] that HNE is formed among many other aldehydes (i.e., pentanal, hexanal, 2-hexenal, 2-heptenal, 2-octenal, 2-nonenal, 2,4-nonadienal, 2,4-decadienal, 9-oxononanoic acid) if an ethanolic solution of 13-hydroperoxy linoleic acid is stored at room temperature in an oxygen atmosphere for 48 h. In this study HNE and the 2,4-alkadienals were identified as the aldehydes which are most toxic to cultured fibroblasts.

The procedure for preparation of HNE from autoxidized lipids in high purity and in quantities required for biological studies proved rather difficult and tedious. Depending on the procedure the yield of HNE from autoxidized linoleic acid, for example, was only in the range of about 1.6 to 3.8 g HNE/100 g; gamma-linolenic acid or arachidonic acid yielded 4.2 and 16.2 g HNE/100 g.[4] To overcome this problem a chemical synthesis which allowed the preparation of 4-hydroxyalkenals of any desired chain length was already developed in 1967.[22] Since then two more methods were published.[23–25]

Most of the studies on 4-hydroxyalkenals performed until the late 1970s dealt with their carcinostatic activity, their potential use for tumor treatment, and their reactivity towards biologically important thiol compounds (glutathione, cysteine, SH-proteins). The aldehyde used most frequently was not HNE but the homolog with 5 carbon atoms, that is, 4-hydroxypentenal, an aldehyde which does not occur in oxidized lipids but was prepared by chemical synthesis.[22] The main reason why this aldehyde was selected was its much better solubility in water (100 mg/mL) compared to HNE (6.6 mg/mL); this was of advantage, for example, in administering the compound to tumor-bearing animals. The large body of information on biological activities of 4-hydroxyalkenals, particularly 4-hydroxypentenal, which accumulated from these early studies were summarized in a book published in 1977[1] and in several reviews published in 1967,[16] 1979,[14] 1982,[2] and 1985.[3] Most of these early studies would have remained largely unheeded by the scientific community without two important additional discoveries. First, the group of Comporti had studied lipid peroxidation in rat liver microsomes for a long time, and they had found that diffusible cytotoxic aldehydes are produced in this process.[10,11,26,27,28] The most cytotoxic aldehyde was finally identified as 4-hydroxynonenal.[29] The discovery

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Table 1. Time Table for Detection of 4-Hydroxyalkenals and Related Aldehydes in Biological Samples and Development of Chemical Synthesis

Schauenstein et al., 1964 (15)	4-hydroxyoctenal is a cytotoxic and carcinostatic substance in autoxidized methyl linoleate. Later (2) it was proven that the aldehyde was in fact HNE.
Esterbauer and Schauenstein, 1966 (34)	4-hydroperoxynonenal in autoxidized methyl linoleate.
Esterbauer and Weger, 1967 (22)	chemical synthesis for HNE and homologous 4-hydroxyalkenals
Erikson, 1974 (23)	chemical synthesis for 4-hydroxyhexenal
Esterbauer et al., 1975 (35)	4,5-dihydroxypentenal is present in heated aqueous deoxyribose solutions
Nakamura et al., 1977 (17)	4-hydroxyhexenal in autoxidized linolenic acid
Benedetti et al., 1980 (29)	HNE, 4-hydroxyhexenal and similar aldehydes are formed by rat liver microsomes in NADPH-dependent, ADP/iron stimulated lipid peroxidation
Paul and Fenical, 1980 (36)	HNE is present in red algae and acute toxic to some fish
Esterbauer, 1982 (2)	HNE is present in autoxidized (ascorbate/iron) linoleic acid, (n-6) linolenic acid, and arachidonic acid
Nakamura and Toyomizu, 1982 (18)	HNE, 4-hydroxyhexenal, 4-hydroperoxyhexenal, 4-hydroperoxynonenal, and 4,5-dihydroxyheptenal in autoxidized linseed oil.
Benedetti et al., 1984 (37)	4,5-dihydroxydecenal in peroxidized rat liver microsomes
Winkler et al., 1984 (38)	HNE in Ehrlich ascites tumor cells treated with an iron histidine complex
Segall et al., 1985 (30)	4-hydroxyhexenal is a hepatotoxic metabolite of the pyrrolizidine alkaloid senecionine
Poli et al., 1985 (39)	HNE, 4-hydroxyhexenal in rat hepatocytes treated with CCl_4 or ADP/iron
Turner et al., 1985 (40)	HNE is a cytotoxic compound present in oil samples collected from the outbreak of the toxic oil syndrome in Spain 1981.
Lang et al., 1985 (41)	HNE is present in various food stuff and oils and in microsomes treated with ADP/iron, CCl_4 or $BrCCl_3$
Ramenghi et al., 1985 (42)	HNE and 4-hydroxyhexenal in red blood cells of thalassemic subjects
Curzio et al., 1986 (43)	HNE in rat pleural exudate
Benedetti et al., 1986 (44)	HNE in mouse liver after bromobenzene intoxication
Oarada et al., 1986 (20)	HNE is a major compound in oxidized methyl linoleate
van Kuijk et al., 1986 (45)	HNE in normal rat liver
Buffinton et al., 1986 (46)	HNE and many other aldehydes in malaria-infected red blood cells
Poli et al., 1986 (47)	HNE in red blood cells of thalassemic subjects
Yoshino et al., 1986 (48)	HNE in plasma and liver of vitamin E deficient rats
Gree et al., 1986 (24)	new synthesis of HNE and other 4-hydroxyalkenals
Tomita et al., 1987 (49)	HNE in plasma and liver of vitamin E deficient rats
Kaneko et al., 1987 (21)	HNE and several 2-alkenals in autoxidized 13-hydroperoxy linoleic acid
Esterbauer et al., 1987 (50)	HNE, 4-hydroxyhexenal, 4-hydroxyoctenal, and some other aldehydes in oxidized human low density lipoprotein
Buffinton et al., 1988 (51)	HNE, hexanal and other aldehydes in malaria-infected red blood cells
Sano et al., 1988 (52)	HNE and hexanal in liver and lung of mice with B_{16} melanoma
De Montarby et al., 1988 (25)	Chiral synthesis of 4(S) and 4(R)-hydroxynonenal (HNE)
Siakotos et al., 1988 (53)	HNE in many tissues of dogs with neuronal retina ceroidosis
van Kuijk, 1988 (54)	HNE in dog retina
Pompella et al., 1988 (55)	HNE in liver of mice following intoxication with allyl alcohol
El Din et al., 1989 (56)	HNE in the wound fluid of posttraumatic patients
Lieners et al., 1989 (57)	HNE in plasma of dogs after hypovolemic traumatic shock
Selley et al., 1989 (58)	HNE in human plasma and monocytes
Tillian et al., 1989 (59)	HNE in Ehrlich ascites tumor cells treated with iron-histidine complexes
Steinberg et al., 1989 (32)	HNE-modified proteins can be detected by antibodies in arteries of Watanabe rabbits and in plasma of rabbits and humans
van Kuijk et al., 1990 (31)	4-hydroxyhexenal in oxidized docosahexaenoic acid
Norsten-Höög and Cronholm, 1990 (60)	HNE in normal rat liver and hepatocytes
Raguenez-Viotte et al., 1990 (385)	HNE in proximal tubular cells of celiptium-treated rats

that HNE is a cytotoxic product of microsomal lipid peroxidation rapidly led to a renewed interest in 4-hydroxyalkenals and initiated a large number of additional investigations related to the generation of HNE by peroxidizing biological samples, its quantitative determination, and its biological activities.

Second, in studies, which were not at all related to lipid peroxidation, it was found by Segall's group[30] that the macrocyclic pyrrolizidine alkaloid senecionine is metabolized in the rat liver by the mixed function oxidase system to 4-hydroxyhexenal, and that the hepatoxicity of these alkaloids is likely mediated by this aldehyde. It is now well established that 4-hydroxyhexenal is also generated during lipid peroxidation due to degradation of omega-3-polyunsaturated fatty acids.[4,31]

A chronological list of studies on chemical synthesis of 4-hydroxyalkenals and similar aldehydes and their detection in various biological systems is given in Table 1. From the many papers published in this field 90% appeared after 1980, the year when it was published that HNE is a cytotoxic product generated during peroxidation of liver microsomal lipids. Of course, a large number of additional publications not listed in Table 1 also exist dealing with the various biological

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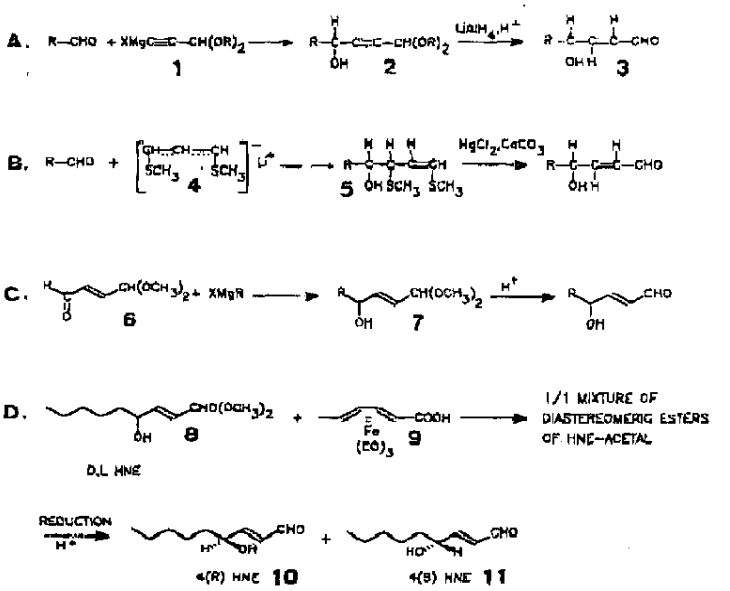

Fig. 2. Methods for chemical synthesis of 4-hydroxyalkenals.

properties of 4-hydroxyalkenals, such as their cytotoxicity, growth inhibiting activity, genotoxicity, chemotactic activity, or potential role in atherogenesis. Recently it has been shown[32,33] that HNE-modified proteins, likely derived from low density lipoproteins, occur in the atheroma of hyperlipidemic rabbits and that autoantibodies against HNE-modified proteins exist in plasma of rabbits and humans. These and the large number of other studies appear to prove clearly that HNE and similar aldehydes are generated in vivo and are of pathophysiological relevance.

Chemical synthesis of 4-hydroxyalkenals

HNE is most frequently synthesized as shown in equation Fig. 2A. The Grignard compound of propynal diethylacetal *1* is reacted with hexanal to 4-hydroxy-2-nonynal diethylacetal 2. The CC triple bond is converted with $LiAlH_4$ at low temperature (−20 °C) to the trans CC double bond and the resulting 4-hydroxy-2-nonenal diethylacetal is then hydrolyzed under mild acidic condition to the free aldehyde, that is, 4-hydroxy-2-*trans*-nonenal *3*. The overall yield of HNE based on the propynal diethylacetal is 53 mol%. The intermediates, that is, hydroxynonynal diethylacetal and hydroxynonenal diethylacetal can be purified by fractional vacuum distillation. The purification of HNE is more difficult since most of it would be destroyed during vacuum distillation. To obtain HNE with a purity of 95% or better, it is therefore important to carefully distill its immediate precursor 4-hydroxynonenal diethylacetal. Alternatively, the free aldehyde can be purified by counter current distribution with chloroform and water[22] or by preparative column chromatography on silicic acid with dichloromethane/hexane 3:1 and by dichlorometh ane/methanol 9:1.[61] The most critical step in the whole synthesis is the conversion of the CC triple bond to the trans double bond. If the temperature during $LiAlH_4$ treatment becomes too high, the diethylacetal group is also attacked and converted into the ethyl-ether (CH_3–$(CH_2)_4$–CHOH–CH=CH–CH_2–OC_2H_5) which is difficult to remove in the subsequent steps. An alternative method to the $LiAlH_4$ reduction is the use of metallic sodium in liquid ammonia.[62,63] Catalytic hydrogenation with Lindlar or other catalysts gives the acetal with the cis double bond, which upon saponification does not give an aldehyde but a 2-pentylfurane.[14,62] When the deuterated or tritiated analogue of $LiAlH_4$ is used, deuterated[58] or tritiated HNE[64] can be obtained. If propanal is used in this synthesis for the reaction with propynal diethylacetal, the end product is 4-hydroxy-2-*trans*-hexenal. Similarly other 4-hydroxyalkenals with different chain lengths were prepared using different *n*-alkanals with different chain lengths.[22,65,66] The 4-hydroxyalkenals with chain lengths up to 13 carbon atoms are liquids at room temperature, those with 14 carbon atoms and more are white crystalline solids.

In 1974 a completely different method for the synthesis of 4-hydroxyhexenal has been described[23] (Fig. 2B). The principle of this method is the reaction of 1,3-bis(methylthio)allyl-lithium *4* with propanal in tetra-

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rahydrofuran cooled to −75 °C to 1,3-bis(methylthio)-1-hexene-4-ol *5*. This compound is then converted with mercuric chloride to 4-hydroxy-2 *trans*-hexenal. The yield was reported to 47%. The bis(methylthio)allyllithium *4* is not a commercial reagent, but must be prepared from 1,3-bis(methylthio)-2-methoxypropane and *n*-butyl lithium. When hexanal is used instead of propanal the resulting 4-hydroxyalkenal is HNE. This procedure was preferentially used by Segall's group[30] for the preparation of 4-hydroxyhexenal or HNE. Substituting non-radio-labeled propanal for tritium-labeled propanal, a 4-hydroxyhexenal with the tritium label at the C-5 position was also prepared.[67] Another method for the synthesis of 4-hydroxy-2-trans-alkenals has been described by Gree et al.[24] (Fig. 2C). Here furan is firstly converted in a two-step reaction to fumaraldehyde monodimethylacetal *6*. This compound is then reacted with *n*-butyl Grignard to 4-hydroxynonenaldimethylacetal *7*. Saponification with 2.5% H_2SO_4 gave 4-hydroxynonenal, the overall yield was reported to be 71%.

Carbon 4 in 4-hydroxynonenal has a chiral center and all three synthetic methods (Fig. 2A,B,C) described above yield a racemic mixture of 4(R)- and 4(S)-hydroxyalkenals *8*. Recently a method for preparing both optically active forms of 4-hydroxynonenal has been described[25] (Fig. 2D). The principle is the esterification of the OH group of 4-hydroxynonenal dimethylacetal with the optically pure chiral iron tricarbonyl complex of sorbic acid *9*. The 1:1 mixture of the two enantiomeric esters were separated by chromatography and Dibal reduction gave 4(R)(−) and 4(S)(+) hydroxynonenal dimethylacetal, which after deacetylation gave the corresponding 4(R)hydroxynonenal *10* (α_D^{25} = −46°, c = 0.45, $CHCl_3$) and 4(S)hydroxynonenal *11* (α_D^{25} = +48°, c = 0.69, $CHCl_3$).

Some physicochemical properties of 4-hydroxynonenal (abbreviations in the literature for this aldehyde are HNE, 4-HNE, 0 4-HN, t-4HN, 9-1-OH) and some of its derivatives used for analysis (Fig. 3) are as follows: 4-hydroxynonenal (ref. 22, and in part unpublished results from the author's laboratory): $C_9H_{16}O_2$; mol. wt. 156.22; n_D 1.471; d_4^{20} 0.944; colorless liquid; soluble in most organic solvents, for example, alcohols, hexane, chloroform, slighdy soluble in water (6.6 g/liter = 42 mM); UV maximum: 223 nm, ε 13750 (in water); 221 nm, ε 13100 (in ethanol); 215 nm, ε 14400 (in hexane); 70 eV mass spectrum: m/e 156 (1%), 86, 85, 71, 57 (100%), 43. Infra red (μ): 2.73, 2.85, 3.44, 3.50, 3.65 (CHO), 5.90 (CHO), 6.07 (C=C), 6.85, 7.25, 8.93, 10.23 (trans C=C). 2,4-dinitrophenylhydrazone *12*:[68] $C_{15}H_{20}N_4O_5$, mol. wt. 336.35, orange crystals melting point 154–155°, UV maximum: 370 nm, ε 28200 (in chloroform); 456 nm, ε 30800 (in ethanolic KOH) 70 eV mass spectrum: m/e 336 (90%), 307, 301, 265, 261, 247, 237 (100%), 203. Semicarbazone *15* (68): $C_{10}H_{19}N_3O_2$, mol. wt. 213.26, melting point 131–134 °C, UV maximum 263 nm, ε 28000 (in ethanol). Trimethylsilylether (TMS) derivative:[41] $C_{12}H_{24}O_2Si$ mol. wt. 228.41, 70 eV mass spectrum: m/e 228 (5%), 213, 199, 184, 157 (100%), 129 (30%), 73 (75%). Pentafluorobenzyloxime trimethylsilylether (OPFB–TMS) derivative *17* (45): $C_{19}H_{26}O_2NSiF_5$ mol. wt. 423.50, 60 eV mass spectrum: m/e 303 (15%), 283 (50%), 167 (40%), 152 (100%). Dimethylhydrazone trimethylsilylether (DMH-TMS) derivative *14*:[20] $C_{14}H_{30}ON_2Si$ mol. wt. 270.49, 25 eV mass spectrum: m/e 270 (90%), 226, 213, 199 (100%), 181, 168, 155, 136, 123, 102, 73 (80%), 44 (25%). Oxime-*t*-butyldimethylsilyl (oxime-tBDMS) derivative *16* (60): $C_{21}H_{45}O_2NSi_2$ mol. wt. 399.82, mass spectrum: m/e 399 (7%), 384, 342 (100%), 328, 284, 268, 210, 149, 147 (65%), 143, 133, 75 (80%), 73 (55%). 5(1′-hydroxyhexyl)-1-methyl-2-pyrazoline (HMP) derivative *13*:[69] $C_{10}H_{20}ON_2$ mol. wt. 184.28, mass spectrum: m/e 184 (9%), 84 (52%), 83 (100%), 56, 42 (86%), 41.

Determination and occurrence

The various methods for determination of HNE and other aldehydic lipid peroxidation products were recently reviewed,[7,8] and therefore we will only discuss them here briefly. In the work[29] leading to the discovery of HNE being one of the major products of microsomal lipid peroxidation, the sample was derivatized with 2,4-dinitrophenylhydrazine (DNPH), and the corresponding hydrazones (Fig. 3, *12*) were analyzed by HPLC. This method was later improved[8] and can be considered as most appropriate if the complete spectrum of aldehydes in a peroxidized biological sample should be analyzed. One should keep in mind that such samples do contain additionally to HNE a great number of other aldehydes (alkanals, 2-alkenals, 2,4-alkadienals, various hydroxyaldehydes). In most of the samples we analyzed HNE and hexanal were the main products.

An alternative HPLC method described[49] for measurement of aldehydes in plasma or liver employs the reaction of aldehydes with 1,3-cyclohexandione (CHD) to yield fluorescent dihydroacridine derivatives, which can be separated by HPLC.

Free HNE, if present in sufficient amounts (more than 2 μM), can easily be measured[41] by HPLC with UV detection at 220–223 nm where it shows a strong absorption maximum. For that, the proteins in the sample are first precipitated by acetonitrile-acetic acid, and the protein free supernatant is injected into an HPLC analyzer. The method is useful in following the

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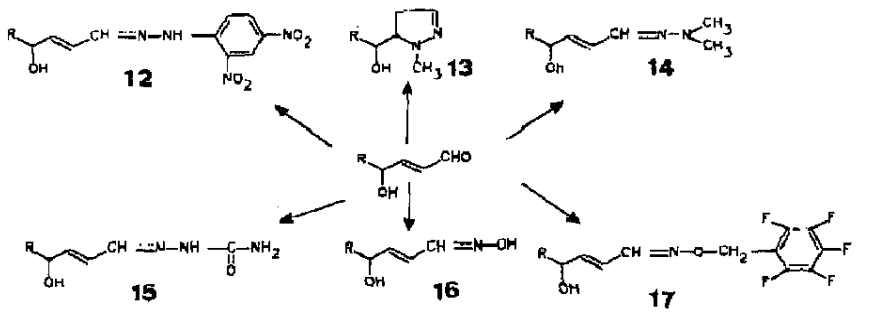

Fig. 3. Derivatives of 4-hydroxyalkenals used for their analysis by HPLC or GC/MS. Compounds *15*, *16*, and *17* are silylated prior to GC/MS.

consumption of exogenous HNE in kinetic experiments (e.g., reactions with amino acids or proteins) or in cell experiments.

Various highly sensitive and specific GC-MS methods are now also available for the determination of HNE. In one method[31,45,58,386] the sample is treated under mild conditions with pentafluorobenzyl (PFB) oxime and then silylated (Fig. 3, *17*). The PFB-oxime TMS derivative is analyzed by GC-MS with negative ion chemical ionization. The fragments specific for HNE have a mass of 152. Quantification of this GC-MS analysis is possible by using deuterated HNE as internal standard[58,386]. The PFB-oxime method is, of course, also applicable for other 4-hydroxyalkenals (e.g., 4-hydroxyhexenal)[31] and other aldehydes. We now use it occasionally for determining the complete aldehyde spectrum in oxidized LDL (unpublished results). In another GC-MS method the sample is derivatized with *N*-methyl-hydrazine.[69] In this reaction HNE gives a 5(1'-hydroxyhexyl)-1-methyl-2-pyrazoline (Fig. 3, *13*), whereas MDA yields 1-methylpyrazole. Both compounds can be separated by GC, and their structure can be confirmed by coupled MS. Recently another GC-MS method for determination of HNE and hexanal in plasma or liver was described[60] (Fig. 3). Here the sample is reacted with hydroxylamine in $HClO_4$. The oximes are then extracted with CH_2Cl_2 and silylated with MTBSTFA to the oxime-*t*-butyldimethyl silyl derivatives, which are analyzed by GC-MS. With this method it was shown that normal rat liver tissue contains 0.55 nmol HNE and 1.26 nmol hexanal per gram. The addition of BHT during sample preparation had only a weak effect on the HNE values (decrease to 0.33 nmol/g), hexanal decreased to 0.68 nmol/g. Another GC/MS method used is reaction with *N*-dimethyl hydrazine to *14* (Fig. 3) and silylation.[20]

A principal problem in determining HNE in biological samples is, of course, the possibility of HNE being formed during the procedures of derivatization and working up by the autoxidation of PUFAS or degradation of oxidation products. To avoid this risk antioxidants (EDTA, BHT) should always be added to the samples. The problem of artifactual HNE is likely to be of minor importance if only its increase in response to an oxidative stress has to be measured and compared to an unoxidized control. Control microsomes, for example, give an HNE value of 0.03 nmol/mg protein. Peroxidized (ADP/Fe) microsomes show 3.4 nmol/mg. Here one could argue about the validity of the control value, but not about the more than 100-fold increase in the peroxidized sample. The situation is similar in many other systems (e.g., low density lipoprotein, hepatocytes) where HNE levels of controls and peroxidized samples were compared. In Table 2 we have listed some of the HNE values reported for plasmas, cells, and subcellular fractions.

Mechanism of formation of HNE and other aldehydes

Possible mechanisms of formation of aldehydes during lipid peroxidation were recently reviewed.[4,6] The main process leading to aldehydes is likely to be the so called β-cleavage reaction of lipid hydroperoxides or, more precisely, the lipid alkoxy-radicals. Thus hexanal arises, for example, by β-cleavage of 15-hydroperoxy-arachidonic acid or 13-hydroperoxy-linoleic acid. The mechanism of the formation of hydroxyalkenals is certainly more complex and not experimentally resolved. From the data on the formation of hydroxy- or hydroperoxyalkenals in model systems (see Table 1) one can conclude that (a) omega-6-PUFAS such as 18:2 and 20:4 can yield 4-hydroxy-nonenal (HNE), 4-hydroperoxynonenal and 4,5-dihydroxydecenal; (b) omega-3-PUFAS as 22:6 can yield 4-hydroxyhexenal, 4-hydroperoxy-hexenal and 4,5-dihydroxyheptenal; (c) omega-9-PUFAS such as 20:3 (present only in traces in tissue) can yield 4-hydroxyundecenal, 4-hydroperoxyundecenal and 4,5-dihydroxydodecenal. With the exception of the latter two all of these aldehydes were found in the respective oxidized PUFAS (Table 1). Possible mechanisms for the formation of HNE from the omega-6-PUFAS were recently proposed by Pryor and Porter.[387] Both suggested mech-

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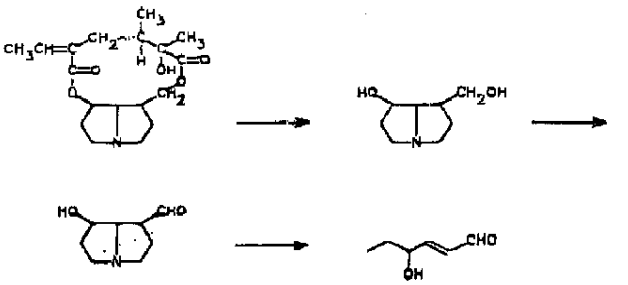

Fig. 4. Metabolic formation of 4-hydroxyhexenal in the liver from the alkaloid senecionine.[30]

anisms meet the requirement that HNE can only be formed from omega-6-PUFAS. For one mechanism the precursor is 11-hydroperoxy-arachidonic acid (or 9-hydroperoxy-linoleic acid), while for the other the precursors would be the positional isomers 15-hydroperoxyarachidonic acid or 13-hydroperoxy-linoleic acid. The mechanism of the formation of 4-hydroxyhexenal from senecionine by the hepatic mixed function oxidase system[30] is shown in Fig. 4.

Chemical reactivity

4-Hydroxyalkenals have three main functional groups, that is, the aldehyde group, the CC double bond and the hydroxy group, which can participate alone or in sequence in chemical reactions with other molecules. Which one of these groups will react depends on the type of reactant and conditions of the reaction. The reaction with thiol compounds has received particular attention, since numerous studies have revealed that 4-hydroxyalkenals added to tissue, cells, or cell fractions cause a rapid loss of SH groups.

Glutathione easily reacts in neutral solutions with 4-hydroxyalkenals.[70] The initial product *18* (Fig. 5) is a saturated aldehyde with the glutathione residue bound by a thio-ether linkage at carbon atom 3. This product then undergoes an intramolecular rearrangement to the five-membered cyclic hemiacetal *19*, which is the main (95%) end product in aqueous solution (Fig. 5A). A similar reaction takes place[71] if a neutral solution of cysteine is mixed with an equimolar amount of a 4-hydroxyalkenal (Fig. 5B), the product is *21* in equilibrium with *20*. If cysteine is present in excess, the aldehyde group of the 1:1-conjugate *20* condenses with a second molecule of cysteine and the end product is a thiazolidine derivative *22*. Other low molecular weight thiols, which have been shown to react with 4-hydroxy-

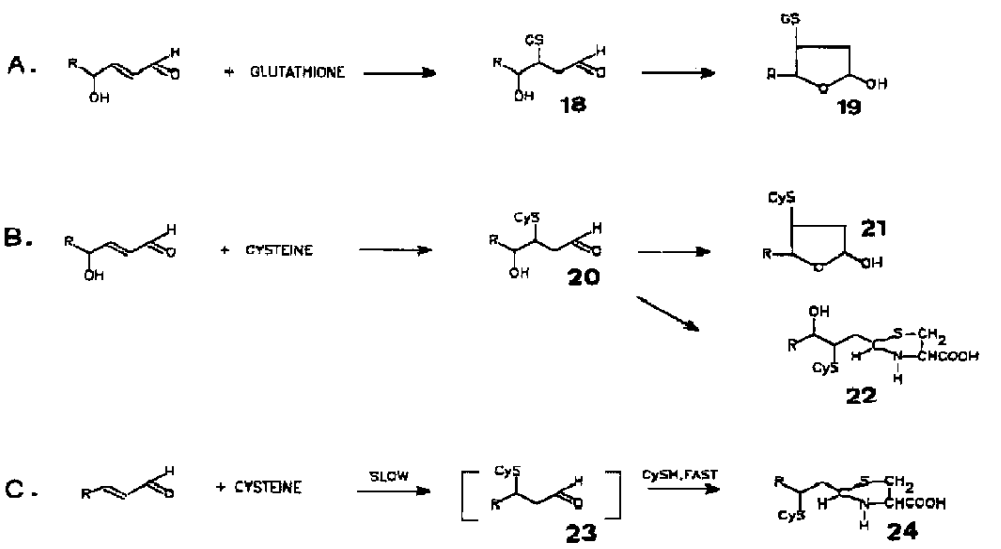

Fig. 5. Reaction of glutathione and cysteine with 4-hydroxyalkenals or 2-alkenals.

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alkenals in the same 1:1 stoichiometry as GSH (Fig. 5A) are thioglycolate, thioglycolic acid ethyl ester,[72] coenzyme A, and *N*-acetyl-cysteine. The reaction of 2-alkenals such as acrolein or crotonal with cysteine proceeds according to the process shown in Fig. 5C. Here the thiazolidine derivative *24* is the only reaction product, because the 1:1 adduct *23* condenses very fast with a second cysteine molecule.

Kinetic investigations[70–73] suggest that such reactions of thiols with 4-hydroxyalkenals and other 2-alkenals are straighrforward without noteworthy side reactions, but nevertheless the detailed mechanism is rather complex and involves the following principal steps:

(1) The dissociation of the thiol RSH into the sulfhydryl anion RS^-:

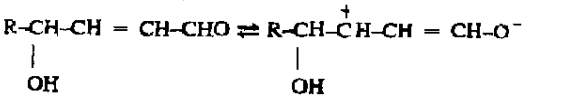
$$RSH \rightleftharpoons H^+ + RS^-.$$

It is always the RS^--form which makes the nucleophilic attack at the aldehyde. The fraction α of the thiol which is present in the reactive RS^--form increases with pH and is given by the equation

$$\alpha = \frac{RS^-}{RSH + RS^-} = \frac{K_{SH}}{K_{SH} + H^+},$$

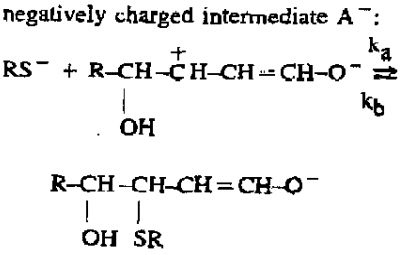
where K_{SH} is dissociation constant of the particular thiol. K_{SH} is in the range of 10^{-7} to 10^{-10} M depending on the thiol.

(2) The delocalization of electrons in the aldehyde in the mesomeric equilibrium:



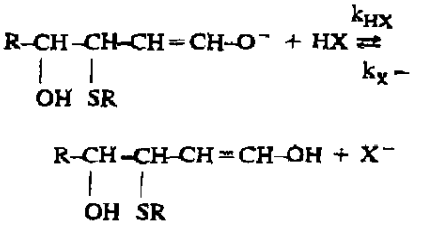
Theoretical conformation studies show that the lowest electron density is at carbon 3.[74]

(3) The nucleophilic addition of the RS^- to the electrophilic center at carbon 3 of the aldehyde yields a negatively charged intermediate A^-:



It has been shown, that the addition of RS^- is the rate-limiting step if the reaction is carried out below pH 4.5.

(4) The negatively charged intermediate reacts subsequently with a proton donor HX (e.g., H_3O^+, H_2O, $H_2PO_4^-$):



At physiological pH and above this is the rate-limiting reaction. It is this step which makes the whole reaction complex and, if the medium is not clearly defined, in fact unpredictable.

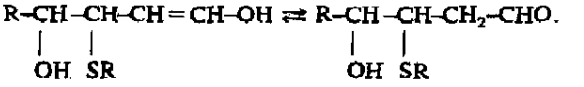
HX can be any substance able to transfer a proton to the negatively charged intermediate. In plain water it would be H_3O^+ and H_2O. At pH 7 and above the H_3O^+ concentration is so low, that its contribution to the proton transfer can be neglected. The rate of reaction 4 is then given by

$$v_{H_2O} = k_{H_2O}\,[A^-][H_2O],$$

where K_{H_2O} is the rate constant for the reaction of water with the negatively charged intermediate. In buffered solution the corresponding buffer acids HX, for example, $H_2PO_4^-$, HPO_4^{--}, CH_3-COOH also participate in the proton transfer. The contribution depends on the concentration HX and the associated rate constant k_{HX}, for example, $v_{H_2PO_4} = k_{H_2PO_4}\,[A^-][H_2PO_4^-]$.

It has been shown,[76,72] for example, that the reaction of α,β-unsaturated aldehydes with thiols at pH 7.0 and above can proceed up to hundred times faster in phosphate-buffered solution than in buffer-free solution at the same pH controlled in a pH-stat. This may also have a significant implication for reactions of α,β-unsaturated aldehydes with SH-groups in proteins, where nearby located histidine residues or a protonated amino-group could perform this proton transfer, which would result in a high reactivity of this particular SH-group.

(5) The enol formed in reaction 4 is rearranged in the keto-enol-tautomerism into the aldehyde:



In reactions with 2-alkenals without the hydroxy-group (e.g., acrolein, crotonaldehyde) this is the last step leading to the final adduct. In case of 4-hydroxy-alkenals cyclization occurs.

(6) The cyclic 5-membered hemiacetal forms in the oxo-cyclo tautomerism:

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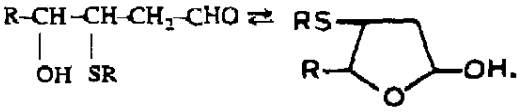

(7) Since all individual steps are reversible the overall reaction is also reversible. The equation for the overall reaction is:

$$\text{2-alkenal} + \text{thiol} \underset{k_2, v_2}{\overset{k_1, v_1}{\rightleftarrows}} \text{adduct.}$$

Some rate constants k_1 and k_2 are given in Table 3.
(8) The rate v_1 of the forward reaction is given by:

$$v_1 = k_1\,[\text{aldehyde}][\text{thiol}],$$

whereby k_1 is the apparent experimentally observed second-order rate constant in $M^{-1}sec^{-1}$. This rate constant changes with pH and the concentration of the proton donors HX according to

$$(9) \quad k_1 = k_a \cdot \alpha \frac{\sum k_{HX}\,[HX]}{k_b + \sum k_x\,[X^-]}; \quad \alpha\text{: see eqn. (1)}, \; k_a, k_b\text{: see eqn. (3).}$$

(10) The rate v_2 of the reverse reaction, that is, the dissociation of the adduct is given by:

$$v_2 = k_2\,[\text{adduct}],$$

whereby k_2 is the apparent experimentally measured rate constant in sec^{-1}. This rate constant also changes with pH ($X^- = OH^-$) and is given by equation (11).

$$(11) \qquad k_2 = k_b \frac{\sum k_x\,[X^-]}{k_b + \sum k_x\,[X^-]}.$$

The equation (9) indicates that the apparent rate constant k_1 should increase with increasing pH and approach a maximum value at a pH, where the thiol is fully dissociated ($\alpha = 1$). This is shown for reactions with glutathione or cysteine in Fig. 6. The absolute rate constants k_a, k_b, k_{HX} were determined for some model reactions.[72] The overall rate constants k_1, k_2 and the equilibrium constant K were determined for various compounds with the C=C–C=O structure (2-alkenals, 4-hydroxy-2-alkenals, α-β-unsaturated ketones) and various thiols.[70–73] A selection of such constants is given in Table 3.

Without going too much into detail such rate constants are very useful in predicting roughly the fate of HNE or other aldehydes produced intracellularly. For

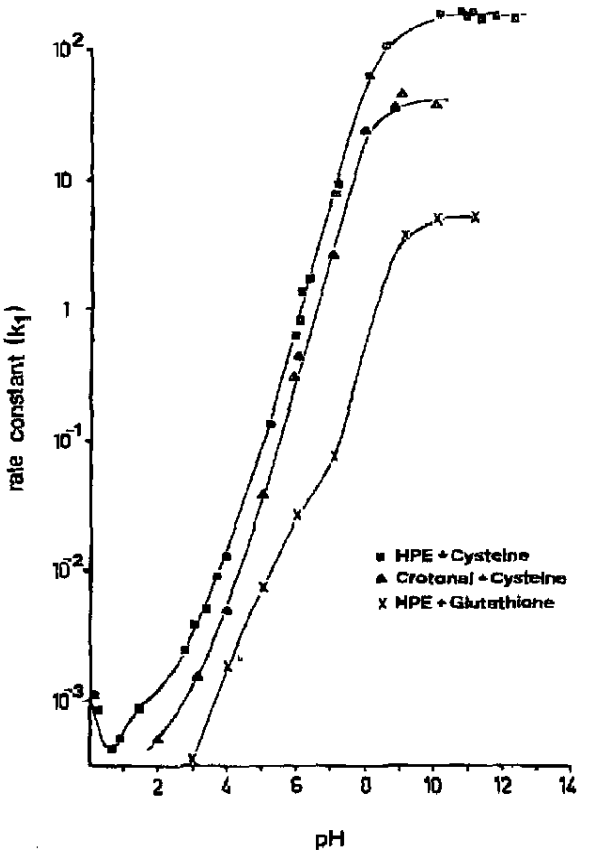

Fig. 6. Effect of pH on the rate of the reaction of α,β-unsaturated aldehydes with thiols. HPE = 4-hydroxypentenal.

example, it follows from the rate constant k_1 for HNE (H 1.09 M^{-1} sec^{-1}), that in the presence of 5 mM glutathione, as it occurs in liver cells, this aldehyde has a half life of only 2 min. Through the action of GSH-transferases, which significantly catalyze this spontaneous conjugation, the half life can of course be further reduced. The HNE concentrations reported for rat liver are in the range of 0.4 to 2.8 nmol/g wet weight (Table 2). Assuming equilibrium conditions for 5 mM glutathione and 1 μM HNE the adduct concentration in liver should be about 20 mM. This is of course unrealistically high and suggests that the HNE-concentration in liver is either much lower than 1 μM, or that the conjugate is rapidly exported.

Clear spectroscopic proofs that the reaction products of thiols with 4-hydroxyalkenals have the structures *19*, *21*, *22* (Fig. 5) were so far only obtained for the products formed in reactions of 4-hydroxy pentenal with glutathione[75] or cysteine.[71] The homologous products formed with 4-hydroxynonenal were not analyzed, but

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one may assume with great certainty that they have identical structures. Structures of the products *24* formed in the reaction of acrolein and crotonaldehyde with cysteine were also analyzed.[71] It is generally assumed, that the reaction of the SH-group of the tripeptide GSH (Fig. 5A) can be seen as a model for the reaction of 4-hydroxyalkenals with SH-groups in proteins. Isolated proteins, which were studied in this respect (again with 4-hydroxypentenal), are glyceraldehyde phosphate dehydrogenase, aldolase, bovine serum albumin, reduced bovine serum albumin (SS-bonds converted to SH), reduced β-lactoglobulin and ovalbumin (for review see 1). From the finding, that the loss of SH-groups corresponded very closely with the number of aldehyde groups newly formed in the protein, as determined with 2,4-dinitrophenylhydrazine, it was concluded that 4-hydroxyalkenals are highly specific reagents which preferentially, if not exclusively, react with SH-groups. In the light of some newer findings[76–82] this assumption, however, should be revised. There is a general problem in the determination of bound aldehydes which arises from the variation of the amount of aldehyde detected with the assay method employed. This dependence is due to variation in the nature and stability of the binding of the aldehyde, which, in turn, affect the result of the assay. Aldehydes may in part be very weakly bound, for example to amino-groups, as carbinolamines (RCH(OH)-NH-protein) and may be fully removed by short washing or dialysis. Aldehydes bound in the form of a Schiff's base to a protein may be split off by weak acids or heating. The thio-ether linkage, which binds 4-hydroxyalkenals or other α,β-unsaturated aldehydes to protein SH groups, as shown for GSH in Fig. 5A, has high stability. This binding is stable under acidic condition and allows the condensation of the free aldehyde group with 2,4-dinitro-phenylhydrazine.[4,7,83] But even aldehydes bound by the thio-ether linkage can be removed from the protein by excess of another low molecular weight thiol. Enzymes with functional SH-groups inactivated by 4-hydroxyalkenals can be reactivated, for example, by excess GSH or cysteine.[83]

That 4-hydroxyalkenals may react with various amino acid residues of a protein was recently demonstrated[77] for the apo-lipoprotein B of the human low-density lipoprotein LDL. In this protein (mol. weight 500.000), HNE (5 mM) modified 45 lysine, 7 histidine, 23 serine, and 51 tyrosine residues, besides the two SH-groups present in this molecule. The chemical structure of the modification is not clear, but it was again found that the binding to lysine is reversible. In view of the ongoing work on polyclonal or monoclonal antibodies directed against aldehyde modified proteins[33,84–86] it would be important to know more about this subject in order to characterize the epitopes recognized by the antibodies. It should be mentioned in this context that

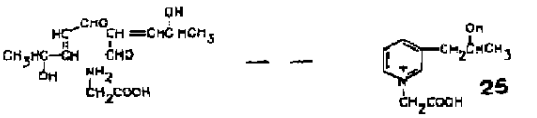

Fig. 7. 4-hydroxypentenal reacts with glycine to the pyridinium derivative *25*. Other products are also formed, they are not yet identified.[76]

some of the aldehyde (for example HNE) modified proteins act as antigen only if they are additionally treated with cyanoborohydride.[33,86] This reagent is specific for Schiff's bases and reduces the C=N-bond to the stable secondary amine bond, for example, RCH_2-NH-protein.

That 4-hydroxyalkenals can react with nearly all amino acids under certain conditions (e.g., slightly alkaline pH of 8.8 and high concentrations of the reactants, in the range of 10 mM) was shown in our laboratory by Napetschnig[78] with 4-hydroxypentenal as model aldehyde. The highest reaction rate (reaction completed in 1 h) as measured by the consumption of 4-hydroxypentenal was with hydroxyproline, somewhat less reactive were Pro, Gly, His, Lys, and Ser. The lowest reactivity (reaction completed in about 10 h) was seen with Ala, Phe, Ile, Arg, Asp, Leu, Asn, Val, Gln, and Glu. The products formed in the reaction with 4-hydroxypentenal with the simplest amino acid, glycine, were further investigated,[76] and one compound (about 20 mol%) could be isolated by preparative HPLC in pure form and identified as a pyridinium derivative, that is, 1-(1-carboxymethyl)-3-(2-hydroxypropyl)-pyridinium betaine *25* (Fig. 7). Several other products were also formed but their instability impeded their isolation. The formation of a pyridinium derivative affords two molecules of the aldehyde reacting stepwise with 1 glycine. A saturated aldehyde (in case of 4-hydroxypentenal it is acetaldehyde) is eliminated by cleavage of the bond between carbon 3 and 4. A possible mechanism of this reaction, which has similarities with the Hantzsch dihydropyridine synthesis, is shown in Fig. 7. The formation of pyridine- and dihydropyridine derivatives was also observed in reactions of primary amino groups (e.g., methylamine, glycine, hexylamine, ethanolamine, polylysine) with malonaldehyde or mixtures of malonaldehyde with monofunctional aldehydes (for review see 87,88 and Figs. 24,25).

That amino groups can react with 4-hydroxyalkenals is also evident from studies related to modifications of deoxy-guanosine. High concentrations of 4-hydroxyhexenal or HNE (1 M) react at 37 °C and pH 7.4 slowly with the deoxyguanosine to give two pairs of diastereomeric adducts *26*.[81] The mechanism proposed (Fig. 8) involves a nucleophilic Michael addition of the NH_2-group of guanosine to the CC double bond of the alde-

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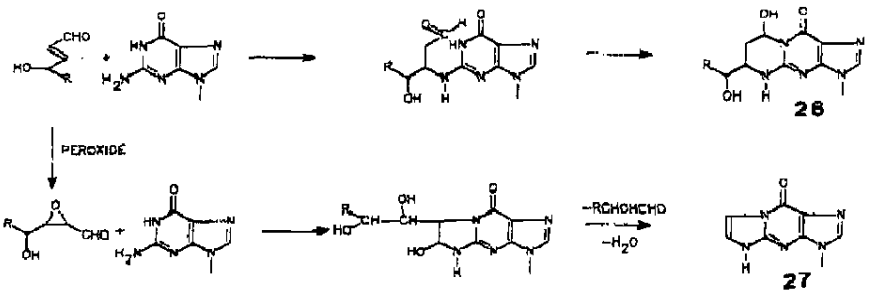

Fig. 8. Reaction of 4-hydroxyalkenals with deoxy-guanosine [81,82]

hyde followed by a rapid cyclization at the 1-N site of deoxyguanosine. In the presence of peroxides a different reaction takes place and the stable end product found with HNE is 1,N^2-ethenodeoxyguanosine *27*; the mechanism proposed[81,82] is also shown in Fig. 8. Similar reactions were reported for the epoxides of crotonaldehyde or acrolein.[89,90] The mechanism illustrated in Fig. 8 assumes that HNE is converted by the peroxide to the corresponding epoxynonanal, which then re- acts to the NH_2-group of guanosine to an intermediate carbinolamine. Cyclization occurs then by reaction of the epoxide ring at the 1-N-site of guanosine. Finally an α-hydroxyalkanal (=2-hydroxyheptanal) and water are split off, and an imidazole ring attached to the pyrimidine base ring is formed. Four additional N^2-substituted deoxyguanosine derivatives, which were also formed in the reaction of HNE and peroxides, and which clearly differed from those formed in the absence of peroxides, could be converted quantitatively to the etheno-deoxyguanosine adduct *27* by increasing the pH of the reaction mixture to 10.5. It was proposed that similar reactions may produce DNA damage in cells which form HNE and peroxides under conditions of oxidative stress.[82] The facile conversion of some of the HNE-guanosine adducts to a single adduct *27* could provide a useful marker for assessing potential DNA damage by 4-hydroxyalkenals produced during lipid peroxidation.

Recently, 2,3-epoxy-4-hydroxynonanal was prepared;[393] This aldehyde exists as a pair of diastereomers and is was shown that it gives in vitro adducts with adenosine, guanosine in a stereoselective manner. Incubation of DNA with the epoxyaldehyde at 37 °C and pH 7.0 yielded modified DNA with about 10 pmol ethenodeoxyguanosine *27* per mg DNA. Contrary to native DNA, single-stranded DNA gave mainly adenine nucleoside adducts. RNA was also extensively modified by the epoxyaldehyde yielding both modified adenine and guanine bases.

Other reactions with NH_2-groups are listed in Fig. 3. They were introduced for characterization and determination of 4-hydroxyalkenals by HPLC or GC/MS. Hydrazines (2,4-dinitrophenylhydrazine, semicarbazide, methylhydrazine) and hydroxylamine condense with the aldehyde group to form the Schiff's base type C=N linkage. Some of these reactions also occur at neutral and more or less physiological conditions, which suggest that similar processes could take place with certain activated amino groups of biomolecules.

General biological effects

Most studies on the biological effects of hydroxyalkenals were done with the intention to elucidate whether they can mediate effects occurring in cells or organs in response to oxidative stress and lipid peroxidation. A compilation of these studies, which were performed with a wide variety of cell types and subcellular fractions, is given in Table 4. In all investigations the aldehydes were added to the respective test system and the effects produced in response to the exogenous aldehyde were then determined after a certain time. The great diversity of effects so far reported can roughly be divided into three categories depending on concentration of the aldehyde:

a. 100 μM and above cause acute and more or less unspecific cytotoxic effects leading in most cases to rapid cell death; many basic catabolic (i.e., mitochondrial respiration) and anabolic (DNA, RNA, and protein synthesis) cell functions are partly or fully inhibited by such high concentrations. Although it seems rather unlikely that HNE or other aldehydes can reach overall concentrations in the range of 100 μM in cells or organs, it is conceivable that such levels may be built up locally near or within peroxidizing membranes for a short time because of their high lipophilicity.[37] It has been calculated, for example, that the concentration of HNE in the lipid bilayer of isolated peroxidizing microsomes is about 4.5 mM,[144] and it has been proposed that HNE might attack critical target proteins within the lipid bilayer. A convincing demonstration for such mechanism has, however, remained elusive so far. On the other hand, if HNE diffuses

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out from the membrane, its concentration will be reduced by the surrounding aqueous phase and the cytosolic HNE-metabolizing enzymes to such an extent that it will no longer be acutely cytotoxic.

b. HNE concentrations in the range of 1 to 20 μM can inhibit DNA and protein synthesis, stimulate phospholipase A_2 and inhibit c-myc expression. A growing number of studies (see Table 2), indicating that HNE may in fact occur at such concentrations, suggest that some of these effects can in fact be produced in tissue in response to oxidative stress.

c. HNE concentrations below 0.1 μM are likely to occur as basal physiological level in many tissues as well as in serum, and effects observed in this concentration range may therefore be of physiological significance. Effects produced by 0.1 μM HNE or less include stimulation of oriented migration of rat neutrophils, that is, chemotaxis,[124–127] modulation of adenylate cyclase activity, weak stimulation of guanylate-cyclase,[139] and stimulation of phospholipase C.[140] Phospholipase C is important for signal transduction in cell membranes mediated by G-proteins. Its stimulation leads to an increased hydrolysis of phosphatidyl-4,5-bisphosphate (PIP_2) with the concomitant formation of diacyl-glycerol (DG) and inositol-trisphosphate (IP_3). DG acts synergistically with IP_3 in the calcium-dependent activation of protein kinase C. IP_3 opens calcium channels, whereas DG strongly increases the calcium affinity of protein kinase C. The protein kinase C plays a prominent role in the control of cell division and proliferation, and in many other cell functions. The stimulation of phospholipase C may also be responsible for the chemotactic effects of 4-hydroxyalkenals since it is known that the chemotactic signal of f-met peptides results in an activation of phospholipase C.[145] Consistent with this assumption is that 4-hydroxyalkenals mediated chemotaxis, and activation of phospholipase C occurred at the same concentration range, and that in both cases the most effective aldehyde was 4-hydroxyoctenal for which the effective doses were extremely low at about 10^{-10} to 10^{-12} M. For HNE the effective doses are 10^{-6} to 10^{-9} M.

The current knowledge of the occurrence of HNE and other 4-hydroxyalkenals in certain tissue (Table 2) and of the effects of exogenous HNE (Table 4) suggests rather strongly that these aldehydes play a pathophysiological and possibly also a physiological role, but one should keep in mind that so far all the evidence for that is indirect. A starting point for direct evidence are the current studies with polyclonal and monoclonal antibodies, which specifically recognize HNE-modified proteins.[32,33,84,86] Immunocytochemical staining with such antibodies convincingly shows that HNE-modified proteins are in fact formed in vivo in animals and man. The application of antibodies for lipid peroxidation studies could be critical in demonstrating the importance of aldehydes as second toxic messengers for oxidative stress and lipid peroxidation.

Cytotoxicity

High concentrations of 4-hydroxyalkenals in the millimolar range are acutely toxic for mammalian cells and lead to cell death within 1 h or less (see Table 5). Preceding or accompanying cell death are a multitude of effects such as rapid depletion of glutathione,[14,91,95,103,107] decrease in protein thiols,[14,94,133] onset of lipid peroxidation,[103,104,132,133] disturbance of calcium homeostasis,[107] inhibition of DNA, RNA, and protein synthesis,[14,93,94,99,106,128,143] inhibition of respiration and glycolysis,[14,94] lactate release,[107] and morphological changes.[1,91] Each one of these effects resembles by its own a severe, but not necessarily an irreversible damage. The time sequence of the onset and development of these effects have not been studied in detail, and clues regarding their relative importance for ultimate cell death are not available. As shown with EATC[91] acute toxic concentrations of HNE (> 1 mM) produce a severe perturbation of the cell surface structure, the microvilli disappear and numerous small blebs appear on the now more or less smooth surface. At this stage the damage appears to be still reversible to some extent, but if the small blebs grow to larger ones, which may even reach the size of the parent cell, the damage is irreversible, and inevitably leads to cell lysis. A similar type of surface blebbing has also been observed in isolated hepatocytes treated with redox cycling quinones (e.g., menadione) and other toxins (for review see 146), and it has been assumed that bleb formation is induced by redistribution of intracellular calcium, which in turn results in an alteration of the cytoskeletal structure. Since HNE also causes disturbances of calcium homeostasis, as shown with isolated hepatocytes[107] or microsomes,[135] and inhibits the assembly of microtubuli,[142] it might be that an impairment of the calcium dependent regulation of the cytoskeletal structure plays a pivotal role in cell death induced by this or similar aldehydes. A disarrangement of the cytoskeletal proteins could also explain the reports that cultured hepatocytes show, after exposure to HNE (100 μM) an altered morphology with a spherical shape and an intense granular structure.[108,110] Richter et al.[388] have recently shown that HNE (10 to 50 μM) inhibits in a concentration-dependent manner prooxidant-induced Ca^{2+} release from mitochondria. This Ca^{2+} release requires oxidation of NADH to NAD^+, hydrolysis of NAD^+ to nicotinamide and mono(ADP-ribose) and a transient binding of mono(ADP-ribose) to

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Table 2. Physiological and Pathological Levels of HNE.

System	Physiological Value	Pathological Value
rat liver (48), nmol/g	2.82 ± 0.53	5.4, vitamin E deficient
rat liver (4), nmol/g	0.48 ± 0.17	
rat liver (60), nmol/g	0.55 ± 0.10	not increased after ethanol administration
rat plasma (48), nmol/mL	0.86 ± 0.20	2.0, vitamin E deficiency
rat hepatocytes (39), nmol/10^8 cells	1.30 ± 0.50	2.7/4.0, after CCl_4 or ADP/Fe treatment
rat hepatocytes (60), nmol/10^8 cells	present*	
rat liver microsomes (41), nmol/mg protein	0.03 ± 0.01	0.2/4.5, after CCl_4 or ADP/Fe treatment
rat retina (54), nmol/g	0.64 ± 0.64	7.7, vitamin E deficiency
dog plasma (57), nmol/mL	< 1.0	45, after reperfusion following hypervolemic traumatic shock
dog, various organs (53)	present*	up to 100 fold more in neuronal retinal ceroidosis
human plasma (4), nmol/mL	0.28 ± 0.34	
human plasma (58), nmol/mL	0.68 ± 0.41	
human low density lipoprotein (50), nmol/mg	0.14 ± 0.17	5.0 to 6.5, in oxidized LDL
human monocytes (58), nmol/10^8 cells	3.9 ± 0.80	
human posttraumatic fluid (56), nmol/mL	1.45	

*not quantified

intramitochondrial proteins. It was shown that HNE inhibits the key reaction, that is, pyridine nucleotide hydrolysis. The consequence would be an overload of mitochondria with Ca^{2+} and an inhibition of Ca^{2+}-dependent mitochondrial enzymes. The cytotoxicity of HNE could therefore in part be due to a disturbance of mitochondrial functions. The time dependence of the onset of cell death after exposure to a single dose of HNE has been determined for EATC.[91, 92] A typical example is shown in Fig. 9.

At the lower concentration range of 10 to 200 μM the cytotoxic effects of HNE become more selective and significant differences exist between different cell types. For some cells, for example, human umbilical vein endothelial cells,[100] fibroblasts,[21,95–98] Chinese hamster ovary cells,[99] doses around 100 μM HNE were lethal and killed all cells in less than 90 min, whereas other cells, for example, EATC (see Fig. 9) or isolated hepatocytes,[13,101] were much more resistant and not lethally damaged by the same dose of HNE. This higher resistance may in part be due to a higher capacity to metabolize and detoxify the aldehyde. On the other hand, methodological differences of cytotoxicity as determined by different laboratories might also be

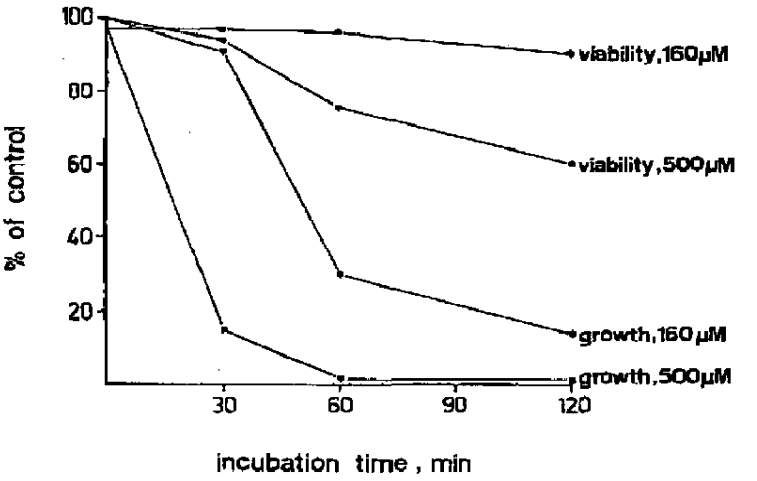

Fig. 9. Time dependence of the effects of HNE on viability and growth capability of Ehrlich ascites tumor cells. A single dose of 160 or 500 μM HNE was added to a suspension of EATC and the viability was determined by trypan blue at the indicated time points. The HNE-treated cells were also reimplanted into mice and the number of tumor cells was determined 7 to 10 days later (curves constructed from values reported in refs. 91, 92).

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Table 3. Rate and Equilibrium Constants for Some Selected Reactions of α,β-Unsaturated Aldehydes with Glutathione (GSH), Cysteine (Cys) and Glycine (Gly).

	k_1 $M^{-1}\,sec^{-1}$	k_2 sec^{-1}	$K = k_1/k_2$ M
Cys + 4-hydroxypentenal	25.0	1.00×10^{-5}	4.0×10^{-7}
GSH + 4-hydroxypentenal	2.71	5.60×10^{-7}	2.9×10^{-7}
Gly + 4-hydroxypentenal	0.01	n.d.	n.d.
Cys + acrolein	220	5.0×10^{-6}	2.3×10^{-8}
GSH + acrolein	121	1.76×10^{-6}	1.4×10^{-8}
Cys + crotonaldehyde	8.90	1.7×10^{-4}	1.4×10^{-5}
GSH + crotonaldehyde	0.78	3.0×10^{-5}	4.2×10^{-5}
GSH + 4-hydroxyhexenal	1.56	4.10×10^{-7}	2.6×10^{-7}
GSH + 4-hydroxynonenal	1.09	9.60×10^{-7}	8.8×10^{-7}
GSH + 2-pentenal	0.47	3.4×10^{-5}	7.4×10^{-5}
GSH + 2-hexenal	0.33	4.7×10^{-5}	1.4×10^{-4}

k_1 is the rate constant for the forward reaction, k_2 for the reverse reaction (see equation 7, page 89). The reactions with GSH and Cys were performed in 0.066 M phosphate buffer pH 7.4, the reaction with Gly at pH 8.8 without buffer. n.d. = not determined.

an important factor why some cell lines are apparently more sensitive than others. In most experiments where HNE showed high toxicity the cell density was low and vice versa. The cell density, of course, determines the HNE-metabolizing capacity of the test system. A summary of toxicity data is given in Table 5. In most of the reports the LD_{50} were not given explicitly, but we have deduced the LD_{50} from dose–effect curves and other data to facilitate the comparison. Kaneko et al.,[21] for example, reported on the cytotoxicity of various aldehydic lipid peroxidation products on human skin fibroblasts (Fig. 10). Supplementation of 3-day cultures

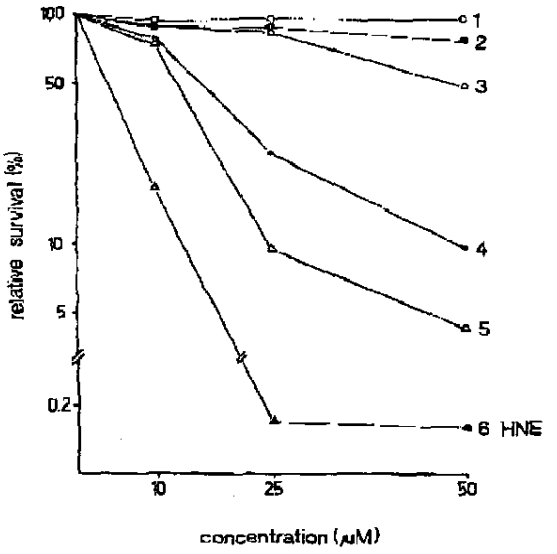

Fig. 10. Toxicity of various aldehydes on growth arrested human fibroblasts. 1: pentanal, hexanal, 2-hexenal, 2-heptenal, 9-oxononanoic acid, 2: 2-octenal, 3: 2-nonenal, 4: 2,4-nonadienal, 5: 2,4-decadienal, 6: 4-hydroxynonenal.

in growth-arrested conditions with 10, 25, and 50 μM HNE induced a severe cell damage, and 24 h later 80, 99, and 99.8% of the cells were dead. The LD_{50} of HNE in this test system was 5 μM. Similar results were found with umbilical vein endothelial cells. Proliferating fibroblasts were somewhat less sensitive (LD_{50} = 18 μM) than growth-arrested fibroblasts. In similar long-term experiments with normal human skin fibroblasts, Poot et al. [95,96,98] found that one addition of 40 μM HNE was not toxic (cells survived for 1 week), but the same dose was lethal for glutathione synthetase deficient skin fibroblasts.[96] This suggests that glutathione depletion together with the capacity of cells to replace glutathione by de novo synthesis are important determinants in the longterm toxicity of low concentrations of HNE. In agreement with that hypothesis, HNE (40 μM) caused only a transient loss (about 70%) of the intracellular glutathione in normal fibroblasts, which was then restored within a few hours, and 24 h after the addition of HNE the glutathione content was even twice as high as in the untreated controls.[95,98]

The importance of cellular metabolism is also evident from studies with H_2O_2-resistent Chinese hamster fibroblasts.[389] These cells adapted and/or selected for a highly peroxidative environment are significantly more resistant as measured by the clonogenic cell survival towards HNE and 2-nonenal than normal fibroblasts. H_2O_2-resistant cells had a 2–3 fold enhanced capacity to remove the aldehyde, probably through the glutathione transferase system.

The dose dependence of the lethal effects of HNE were also determined on Chinese hamster ovary cells (LD_{50} = 100 μM, 1.5 h) and human umbilical vein endothelial cells (LD_{50} = 25 μM, 3 h). Kaneko et al. studied structure–activity relationships and determined for a number of lipid peroxidation products the cytotoxicity towards cultured human fibroblasts[21] and hu-

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Table 4. In Vitro Effects of 4-Hydroxyalkenals.

Text System	Effects Observed[1]	Dose Range[2] μM	Aldehydes[3] Tested	References
Ehrlich ascites tumor cells, peritoneal	formation of blebs, lysis and cell death, 1 h	1000–2000	5,9	1,91,92
	LD_{50}, 2 h	500	9	91,92
	growth inhibition after i.p. reimplantation into mice	150–250	9>5	91,92
	inhibition of DNA synthesis, thymidine incorporation	50–100	9>6	14,93,94
	inhibition of protein synthesis, leucine incorporation	140	5	94
	inhibition of anaerobic glycolysis	150–250	8>5	14,94
	inhibition of RNA synthesis	320	5	14,94
	depletion of glutathione	300	5,9	14,91
	inhibition of respiration with glucose as substrate	300–500	8>5	14,94
	50% loss of cellular protein SH groups	1000	5	14,94
	inhibition of glyceraldehyde-P-dehydrogenase	150–250	5	1
Ehrlich ascites tumor cells in culture	lysis and cell death, LD_{50}, 2 h	100	8>9>5	93
	LD_{50}, 45 h	14–24	8>9>5	93
	growth inhibition in culture after 45 h	7–20	9>8>6	93
	inhibition of DNA synthesis	20	9	93
fibroblasts	cell death, quiescent fibroblast more sensitive than proliferating	5–50	9	21,95,96,98
	colony-forming efficiency	2	9,8,5	97
	disturbance of cell cycle, arrest in G_1 and G_2	2–20	9	96
	inhibition of DNA synthesis	8	9	98
	inhibition of protein synthesis	18	9	98
	decrease of glutathione followed by a two-fold increase after 24 h	20–40	9	95
	inhibition of O^6-methylguanine DNA methyltransferase	100	5>9>8	97
Chinese hamster ovary cells	lysis and cell death, LD_{50}, 1.5 h	100–170	8>9.6	99
	decrease in colony-forming efficiency	20–60	9,8,6,5	99
	genotoxic effects (see Table 8)	10–60	9,8,6,5	99
endothelial cells	lysis and cell death, LD_{50}, 3 h	25	9	100
	inhibition of cell growth	5–10	9	100
rat hepatocytes	lysis and cell death, 1 h	500–1000	9	101,102
	90% depletion of glutathione in 3 min, 2×10^6 cells/mL	1000	9	103
	strong enhancement of chemiluminescence and pentane formation, TBARS	2000	9	103,104
	inhibition of protein synthesis	250	9	105
	inhibition of microsomal glucose-6-phosphatase	250	9	105
	destruction of cytochrome P_{450}	250	9	105
	inhibition of thymidine transport	780	9	106
	disturbance of calcium homeostasis, depletion of glutathione, ATP and NADH, LDH release	480	6	107
	various genotoxic effects (see Table 8)	0.1–600	9,6	108,109,110
	inhibition of triglyceride secretion	100	9	111
	protein kinase C inhibition	100	9	112
	induction of heat shock protein HSP 31	100–200	9	113
	rapidly metabolized to glutathione conjugate, carboxylic acid and 1,4-dihydroxynonene	100–1000	9	13,114,101
Chinese hamster V79 cells	decrease of colony-forming efficiency	30–45	9	115
	increase of mutation (see Table 8)	20–45	9	115
Yoshida ascites hepatoma cells	inhibition of DNA synthesis, thymidine incorporation	40	9	106
	inhibition of DNA polymerase α and β	210–460	9	106
	inhibition of thymidine transport	170	9	106
	inhibition of S-adenosyl methionine decarboxylase	100	5	111
rat hepatoma MH_1C_1 cells	metabolized in 5 min by 3.9×10^6 cells/mL	100	9	116
	induction of a 31 kDa heat shock protein	100–200	9	113
human platelets	potentiation of ADP, thrombine, and ionophore induced aggregation	10–100	9	117
	stimulation of phospholipase A_2	10–100	9	117
	inhibition of ADP and arachidonic acid induced aggregation	300–600	9	118
	80% metabolized in 30 min	20–100	9	118

(continued)

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Table 4. Continued

Text System	Effects Observed[1]	Dose Range[2] μM	Aldehydes[3] Tested	References
HeLa cells	induction of a DNA binding protein, similar to heat shock factor HSF	150	9	119
erythrocytes	lysis	450		29
	decreased resistance to osmotic stress	500–1000	9,6	120
erythro-leucemic cells	reduction in c-myc transcription	0.1–10	9	121
pulmonary macrophages	decrease of membrane fluidity	50–100	9	122,123
	decrease in O_2^- -production			
rat neutrophils	chemotactic, stimulation of oriented migration	less than 1	8>9	124,125,126
	inhibition of migration	100		
human neutrophils	stimulation of random migration, chemokinetic	1	9	127
	inhibition of migration	100	9	
malaria parasite	inhibition of DNA synthesis, that is, hypoxanthine incorporation, toxic	50	9	128
Physarium polycephalum	delay in onset of mitosis	1000–4000	5	129
Salmonella typhimurium	cell death, that is, decrease of survivals	1000–2000	9>8>5	130
	mutagenic (see Table 8)		5	130
rat liver microsomes	inhibition of glucose-6-phosphatase	100–200	9,5	29,131
	destruction of cytochrome P_{450}		9	29
	stimulation of lipid peroxidation by reduction of glutathione dependent protection	100–1000	9	132,133
	formation of fluorescent (430 nm) chromolipids in presence of NADPH or ADP-iron	770–1500	9	134
	disappearance of HNE (50 μM, 1 mg protein/mL) in 1 h	50	9	131
	inhibition of calcium sequestering activity	42	9	135
mouse liver microsomes	conversion of cytochrome P_{450} to P_{420}	1000–2000	9	67
	covalent binding to protein	100–2000	6	67
rat liver mitochondria	inhibition of respiration	200	9	111
	inhibition of succinate dehydrogenase	500	5	136
	inhibition of ATPase and phosphate transport	above 1000	5	137
	formation of fluorescent (430 nm) chromolipids in presence of ADP-iron	770–1500	9	131
rat liver plasma membrane	stimulation of adenylate cyclase followed by inhibition	1–10	9	139
	weak stimulation of guanylate cyclase	0.05	9	139
	inhibition of calcium ATPase	1000–2000	9	139
	inhibition of 5'-nucleotidase	1300–5000	9	139
	stimulation of phospholipase C (PiP_2 hydrolysis)	less than 1	8>6>9	140,141
rat lung membrane	inactivation of beta-adreno receptor	500–2500	9	63
bovine brain microtubuli	inhibition of polymerization	100–1000	9	142
rabbit reticulocyte lysate	inhibition of protein synthesis	480	9	143

1. The time indicates when the effect was measured. For example, in case of peritoneal Ehrlich ascites tumor cells incubation with 500 μM HNE leads to 50% cell death (LD_{40}) within 2 hs; LD_{50} determined by trypan blue exclusion.
2. Given is the approximate range of concentrations tested, if only one value is given it is the approximate half maximal dose.
3. The figures give the chain length of the 4-hydroxyalkenals used in the assay; 9 > 5 means that 4-hydroxynonenal was more effective than 4-hydroxypentenal.

man umbilical vein endothelial cells;[100] a typical example is given in Fig. 10. Saturated aldehydes (pentanal, hexanal, oxo-nonanoic acid) were not toxic up to 50 μM, the highest dose tested. 2-alkenals (heptenal, octenal, nonenal) were toxic at 50 to 100 μM. 2,4-alkadienals (nona-dienal, deca-dienal), HNE and 13-hydroperoxylinoleic acid were highly toxic with LD_{50} in the range of 10 to 25 μM. In a homologous series of aldehydes (e.g., 2-alkenals, 2,4-alkadienals), the cytotoxicity increased with increasing chain length of thealdehydes, which indicates that additional to the general structure the lipophilicity is critical for the cy-

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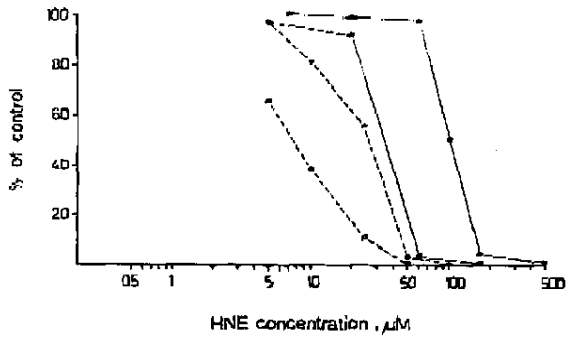

Fig. 11. Dose dependence of the effect of HNE on viability and proliferation of Chinese hamster ovary cells (solid line), and umbilical vein endothelial cells (dotted line). ● viability, ■ proliferation.

totoxic properties of an aldehyde. Lipophilic aldehydes will be mainly localized in the cellular membrane system; this could promote inactivation of membrane-bound enzymes, and furthermore localization in the membranes would be likely to protect the aldehydes from rapid detoxification by cytosolic enzymes.

Effects on cell proliferation

HNE and other 4-hydroxyalkenals possess the capacity to block cell proliferation at concentrations where lethal effects are low or absent. Fig. 9 shows that EATC exposed to 160 μM HNE for 2 h were fully viable (no trypan blue cells present), but proliferation of the cells after reimplantation into mice was reduced by about 85%. A significant inhibition of cell proliferation by HNE was also reported for Chinese hamster ovary cells,[99] umbilical vein endothelial cells [100] (Fig. 11), Chinese hamster V79 cells,[115] human bronchial fibroblasts,[97] and human skin fibroblasts.[96] In this context it should be mentioned that the carcinostatic activity of 4-hydroxyalkenals is well established, and various transplanted animal tumors could be cured by these aldehydes without toxic side effects (for review see 1). Reasons which were offered to explain the growth inhibition include the HNE-mediated inhibition of the DNA polymerase system[106] and a disturbance of the cell cycle.[96] DNA polymerase α is an SH enzyme, which can be blocked by sulfhydryl reactive agents including α,β-unsaturated aldehydes, such as HNE or acrolein. Isolated DNA polymerase α is, however, rather resistant to HNE (ID_{50} = 210 μM),[106] and it is therefore unlikely, that inhibition of cell proliferation and overall synthesis of DNA,[14,93,98] which occurs at much lower concentration, results from inhibition of the DNA polymerase system.

In human fibroblasts[95] HNE concentrations of 2 to 20 μM caused a dose-dependent arrest of cells in the

Table 5. Cytotoxicity of HNE Towards Different Cell Types.

Cell Type	LD_{50}, Time
isolated rat hepatocytes (101, 102)	1000 μM, 1h
culturated rat hepatocytes (108, 110)	100 μM, 20 h
intraperitoneal Ehrlich ascites tumor cells (91, 92)	500 μM, 2 h
culturated Ehrlich ascites tumor cells (93)	100 μM, 6 h
Chinese hamster ovary cells (99)	100 μM, 1.5 h
umbilical vein endothelial cells (100)	25 μM, 3 h
human skin fibroblasts (95, 98)	> 40 μM, 1 week
glutathione synthetase deficient skin fibroblasts (95)	20 μM, 1 week
fibroblasts in logarithmic growth (21)	18 μM, 20 h
fibroblasts growth arrested (21)	5 μM, 20 h

G_2-phase of the first cell cycle, and in the G_1-phase of the second cell cycle. Additional effects were a delay in onset of proliferation due to a retardation of the transit of the cells from G_0 to G_1, and a prolongation of the G_1-phase. This severe disturbance of the cell cycle explains that HNE-treated cells show a diminished DNA synthesis[14,93,94,106,128] and a diminished rate of proliferation. The underlying molecular mechanisms leading to this severe disturbance of the cell cycle, however, remain to be established. In view of some newer findings[113,114,121] it seems reasonable to assume that HNE affects cell proliferation by interfering with the expression of certain genes. In erythroleukemic cells 0.1 to 10 μM HNE caused a dose-dependent reduction of the c-myc oncogen transcription.[121] The c-myc protein seems to be involved in the regulation of cellular multiplication. HNE has also the capacity to induce heat shock proteins[113] and a DNA-binding protein similar to the heat shock factor HSF,[119] which further suggests that some biological effects of HNE may be mediated by altered gene expression.

Similarly to the cytotoxic activity, the antiproliferative activity depends on the structure of the aldehyde and its lipophilicity. Kaneko et al.[100] showed that the inhibition of cell proliferation is absent or low in alkanals. More effective are 2-alkenals, and the most powerful inhibitors are 2,4-alkadienals and HNE. With Chinese hamster ovary cells the inhibition of plating efficiency was determined for a series of 4-hydroxyalkenals with chain lengths from 5 to 11 carbon atoms.[99] The efficiency increased proportionally to the chain length from ID_{50} = 80 μM (for 4-hydroxyhexenal) to an ID_{50} of about 5 μM for 4-hydroxyundecenal.

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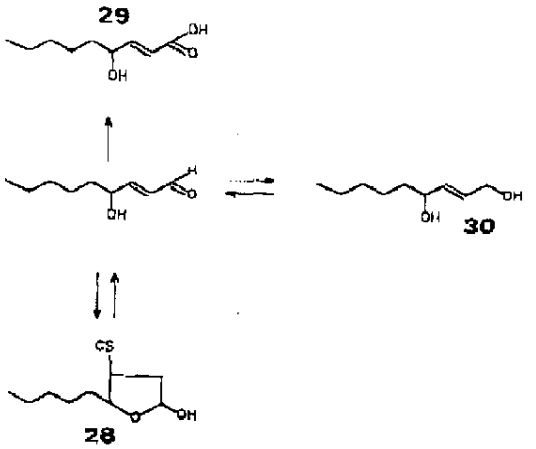

Fig. 12. The main metabolites of HNE formed in the liver are the glutathione conjugate *28*, the 4-hydroxy-2-nonenoic acid *29* and the 1,4-dihydroxynonen *30*.

Metabolism of HNE

The metabolism of HNE, 4-hydroxyhexenal, and 4-hydroxypentenal was studied in vitro with hepatocytes,[13,114] hepatoma cells,[116] isolated rat heart,[73] and in vivo in rats.[67,147] These studies were supplemented by in vitro experiments with subcellular fractions of rat liver cytosol, microsomes, and mitochondria as well as purified enzymes (aldehyde dehydrogenases, alcohol dehydrogenase, and glutathione transferases). The current knowledge (for review see 13) indicates that the main enzymes involved in the metabolism are glutathione transferases, aldehyde dehydrogenases, and alcohol dehydrogenases. The main primary metabolites of HNE are therefore (Fig. 12) the glutathione conjugate *28*, the corresponding carboxylic acid, 4-hydroxy-2-nonenoic acid *29*, and the corresponding alcohol 1,4-dihydroxy-nonen *30*. HNE metabolizing aldehyde dehydrogenase isoenzymes are present in the hepatic cytosol, mitochondria, and probably also in microsomes.[148–153] The HNE-metabolizing NADH-dependent alcohol dehydrogenase is localized mainly in the hepatic cytosol.[101] The glutathione transferases are ubiquitous enzymes, with again the liver being particularly rich. Among the different isoenzymes, rat liver glutathione transferase 8-8 has by far the highest specificity for HNE (66, for review also see 13). Among human GSH transferases the isoenzyme μ shows the highest activity with HNE. Mouse liver GSH transferase only shows weak activity with HNE. As discussed previously HNE readily reacts with GSH also in a nonenzymatic reaction. The GSH transferase-catalyzed reaction can, however, proceed about 300 to 600 times faster. Danielson et al.[66] have proposed that some of the GSH transferases may have evolved specifically for detoxification of HNE and similar aldehydes resulting from lipid peroxidation. The HNE-GSH adducts exhibit a feedback inhibition on the GSH transferases. Isoenzyme 4-4, for example, is inhibited by 5 μM HNE-GSH adduct to 50% with 1-chloro-2,4-dinitrobenzene as substrate. HNE is now used very frequently as substrate to characterize the various types of GSH-transferases (see, e.g., ref. 390). In rats, liver has by far the highest capacity to metabolize HNE.[101] Rat hepatocytes (10^6 cells/mL) were able to metabolize about 90% of 100 μM HNE within 3 min.[101] The approximate product distribution seen after 3 min was 25% HNE-GSH adduct *28*, 25% 4-hydroxynonenoic acid *29*, and 10% 1,4-dihydroxynonen *30*.[13] After longer incubation, dihydroxynonen nearly completely disappeared, whereas the levels of the other two metabolites remained more or less constant.[154] A number of other metabolites were also formed in minor quantites, but their structure has so far not been identified. In the whole animal the HNE-GSH conjugate is exported from the liver and further metabolized to a mercapturic acid. This has been experimentally proved with tritium-labeled 4-hydroxyhexenal.[147] After injection of this aldehyde into the portal vein of rats, within 8 h about 50% of the radioactivity was excreted in the urine as the C-3 mercapturic acid conjugate of 4-hydroxyhexenal. A substantial amount of radioactivity was also bound to proteins, but not DNA or RNA. Feeding a fraction of oxidized linoleate rich in 14-C labeled HNE to rats also resulted in an excretion of radioactivity in urine.[20] Additionally a substantial fraction of the radioactivity was expired as radioactive CO_2, indicating that the aldehyde was metabolized by mitochondria, most likely through β-oxidation of the 4-hydroxynonenoic acid.

Effects on isolated organs and whole animals

In the perfused rat heart administration of 50 μM of HNE for a period of 2 min caused an instantaneous increase in the coronary flow which returned to its original level when the HNE was washed out.[155] This coronary vasodilation was not accompanied by an increase in total heart cyclic GMP or AMP and therefore a cyclic nucleotide independent mechanism must have been involved. In addition, it was found in experiments with the perfused rat heart that HNE is rapidly conjugated with glutathione by a cardiac glutathione transferase and the resulting glutathione S-conjugate is exported by a carrier-mediated process into the perfusate.[73] Prolonged perfusion (> 15 min) led to a depletion of intracellular glutathione and a progressive decrease in the peak systolic pressure of the left ventricle.[73] Treatment of the isolated left atria from rat heart with HNE (10–100 μM, 25 min) led to a reduction in the

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Table 6. Some Selected Studies on the Occurrence of MDA (TBARS) in Biological Samples.

Author	Method of Generation	Method of Measurement	Ref.
Scherz 1968	Irradiation of glycerol solutions	Methylindole	203
Scherz and Stelik, 1968	Irradiation of glucose solutions	Methylindole	172
Scherz, 1970	Photolysis of solutions of carbohydrates	Methylindole	173
Scherz, 1972	Irradiation of foodstuffs	Methylindole	171
McMillan et al., 1978	Thromboxane synthesis in platelets	TBA cell free supernatant	176
Satoh, 1978	Cerebrovascular disorders, serum	TBA, TCA precipitate	208
Gutteridge, 1979	DNA damage by bleomycin	TBA	286
Halman and Bloch, 1979	UV irradiation of formaldehyde solutions	TBA	263
Bragt et al., 1979	Kaolin-induced granulomatous inflammation, rat, plasma, liver	TBA	264
Sato et al., 1979	Diabetes human plasma	TBA	220
Santos et al., 1980	Stroke human plasma	TBA	218
Bond et al., 1980	Cervical mucus stimulated by Cu intrauterine device, but see 128	polarographically	266
Lee, 1980	Storage of plasma	TBA, total plasma	219
Burger et al., 1980	Degradation of DNA by Fe(II) Bleomycin	TBA	267
Gutteridge and Thomas, 1980	Autoxydation of candicidin	TBA	268
Salerno et al., 1981	Ergonovine induced coronary spasm, platelets	TBA	269
Yasaka et al., 1981	Ingestion of paraquat, human serum	TBA	214
Gutteridge, 1981	Free radical damage of amino acids	TBA	270
Loeper et al., 1983	Atherosclerosis, human serum	TBA	265
Aznar et al., 1983	Myocardial infarction human serum	TBA, total serum	215
Rao et al., 1983	Ischemia and reperfusion		271
Hiramatsu et al., 1984	Thermal injury, human serum	TBA	272
Hirayama et al., 1984	Vegetable oil	HPLC of a dansylhydrazin derivative	180,181
Draper et al., 1984	Vitamin E deficiency, rat urine	HPLC	175
Alleman et al., 1985	Hexachlorobenzen-induced porphyria, rat liver	TBA	274
Maiorino et al., 1985	Endotoxin shock, rat liver		275
Schulte-Frohlinde and von Sonntag, 1985	Radiolysis of thymidine		276
Janero and Burghardt, 1986	Xanthine-oxidase iron promoted per oxidation of cardiac membranes	TBA	277
Jeny et al., 1986	Human experimental atheroma, plasma	TBA	279
Mahanty et al., 1986	Aspirin stimulates MDA formation in mouse microsomes	TBA	280
Schimke et al., 1986	Myocardial infarction, human serum	TBA Yagi	281
Quash et al., 1987	Enzymatical formation from spermine	TBA paper chromatography	225

β-adrenoreceptor function, and reduction of contractive force followed by a complete contraction failure.[156] A similar effect was also observed with rat diaphragm (see ref. 1, p. 86). Inactivation of β-adrenoreceptors by HNE (0.5 to 2.5 mM) was also observed in isolated lung membranes.[63]

4-Hydroxyhexenal injected as aqueous solution intraperitoneally into rats caused severe localized necrotizing peritonitis.[30] On the other hand, 4-hydroxyhexenal injected intravenously in form of a phospholipid emulsion (13–18 mg hydroxyhexenal/kg body weight) caused liver damage very similar to carbon tetrachloride poisoning and senecionine poisoning. It was therefore concluded[30] that hydroxyalkenals are involved in the mechanism by which these two toxins cause liver damage.

Mice which orally received an HNE-rich fraction of oxidized linoleic acid showed severe lymphocyte necrosis in the thymus 24 h later.[157] The LD_{50} of HNE for mice is 0.44 mmol = 68.6 mg/kg body weight if given intraperitoneally. The LD_{50} values determined for other 4-hydroxyalkenals are 1.46 (4-hydroxyoctenal), 1.22 (4-hydroxyheptenal), 0.98 (4-hydroxyhexenal), and 0.99 (4-hydroxypentenal) mmol/kg body weight (see ref. 1, p. 54).

In one study not concerning lipid peroxidation,[36] HNE was found to be one of the most fish-toxic principles in some red algae. For reef-dwelling fish (e.g., Eupomacentrus leucosticus) a dose of 8 μg HNE/mL was acutely toxic. During the outbreak of the toxic Spanish oil syndrome (midth 1981) a connection has also been proposed between the HNE contents in oil samples collected and the toxicity of these oil samples. A bioassay-screening program gave correlation between the toxicity of the oil samples and their HNE contents.[40] Rats with Yoshida hepatoma A120 showed a disturbance of the cell cycle in the tumor cells[111] after administration of 25–50 mg HNE/kg body weight. A

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Table 6. Continued

Author	Method of Generation	Method of Measurement	Ref.
Tomita et al., 1987	Vitamin E deficiency, rat plasma, rat liver		49
Mukhopadhyoy et al., 1988	Platelets treated with Cd		282
Skomyakov et al., 1988	Craniocerebral injuries, CSF, human	TBA	283
Uamo et al., 1988	Photoirradiated corn oil	GC	189
Ekström et al., 1988	$CHCL_3$ ingestion, rat, urine	HPLC of DNPH derivative	186
Cheeseman et al., 1988	Hydroxyl radical induced degradation of 2-deoxyribose	HPLC	284
Sönnerborg et al., 1988	HIV infection	Yagi	285
Lieners et al.	Hypovolemic traumatic shock canine plasma	TBA, total plasma	57
Vendemiale et al., 1989	Alcohol ingestion, human plasma	TBA-HPLC,total plasma	287
Ferrali et al., 1989	Allyl alcohol ingestion, mice plasma	TBA TCA supernatant	288
Honjo et al.,1989	Acute pancreatitis, human serum	Yagi	289
Tomita et al., 1989	Ovariectomy, rats plasma	Yagi	273
Albano et al., 1989	Incubation of hepatocytes with alcohol	TBA, TCA supernatant	290
Dehnis and Shibamoto, 1989	UV-irradiation squalene	GC	278

Table 7. MDA or TBARS Values Reported for Normal Human Plasma.

MDA nmol/mL	Reaction Conditions	Ref.
3.4–4.0	TCA precipitate of serum was reacted with TBA/Na_2SO_4 for 30 min at 100°C	208
3.3	Phosphotungstic acid precipitate of serum was reacted with TBA/acetic acid for 60 min at 95°C	209
3.8–4.6	TCA precipitate of plasma was reacted with TBA/Na_2SO_4 for 30 min at 100°C	210
0.94	Plasma was reacted with TBA/HCl/TCA for 30 min at 100°C	211
0.9–1.88	Phosphotungstic acid precipitate of serum was reacted with TBA/acetic acid for 60 min at 95°C	212
3.92	Phosphotungstic acid precipitate of serum was reacted with TBA/acetic acid for 60 min at 95°C	213
0.6	Total plasma was reacted with phosphoric acid/TBA for 60 min at 100°C and determined by HPLC	199
3.74	Phosphotungstic acid precipitate of serum was reacted with TBA/acetic acid for 60 min at 95°C	214
47.2	Plasma was reacted with TBA/HCL/TCA for 30 min at 100°C	215
35.1	Plasma was reacted with TBA/HCL/TCA for 30 min at 100°C	218
0.61		217
0	Deproteinized plasma was separated by HPLC and peak absorbance measured at 267 nm	207
1.7		216
0.32	Plasma was reacted with TBA for 90 min at 60°C	219

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great number of studies were published on the potential use of 4-hydroxyalkenals and some of their derivatives for treatment of experimental animal tumors (for review see ref. 1, pp. 75–81).

Other effects

In a series of papers, Curzio et al.[124–127] showed that 4-hydroxyalkenals possess chemotactic activity towards rat neutrophils, and induce oriented migration and morphological polarization in concentrations between 10^{-6} to 10^{-12} M depending on the chain length of the aldehyde. The most effective aldehyde was not HNE but 4-hydroxyoctenal having its chemotactic peak at 10^{-11} M. 2-Alkenals (e.g., 2-nonenal) also exhibited chemotactic activity in this test system, whereas alkanals were ineffective indicating that the CH=CH–CHO grouping is an essential structural requirement. In this context it should be mentioned that this functional group is also present in the chemotactic C12 aldehyde, 12-oxododeca-5,8,10-trienoic acid, a major lipoxygenase metabolite formed in neutrophils from arachidonic acid.[158] In human neutrophils HNE (0.01–1.0 μM) stimulated random migration (chemokinesis), and a chemotactic response was also observed in some neutrophil preparations.[127] A specific receptor for HNE in neutrophils has not been identified so far, and in view of the finding that HNE and other 4-hydroxyalkenals stimulate phospholipase C[140,141] as do other chemotactic compounds such as f-met-peptides,[143] it is reasonable to assume that chemotaxis is caused by an HNE mediated activation of phospholipase C. Since HNE, and probably also other aldehydes, are formed during the acute phase of inflammation they may well play a role as mediators in the inflammatory processes.

HNE in the concentration range of 10 to 100 μM potentiated platelet aggregation and thromboxane A_2 formation.[117] Since the release of arachidonic acid from the platelets was also increased, it was concluded that HNE can stimulate phospholipase A_2 activity. High concentrations of HNE (350 to 600 μM) have the contrary effect and inhibit ADP or arachidonic acid-induced platelet aggregation.[118]

Another interesting biological effect of HNE is its ability to effect heat shock gene expression.[113,119,159] In rat hepatocytes and rat hepatoma cells MH_1C_1, HNE (100 to 200 μM) induced the synthesis of a 31 kD protein in a dose dependent manner and, to a lesser extent, a 70 kD protein belonging to the heat shock protein (hsp) family.[113] In heat-shocked hepatocytes (2 h, 42°C) the identified hsp were at 100, 95, 80, 70, 47, 31, 27, and 24 kD. In heat shocked MH_1C_1 cells the hsp were 100, 85, 80, and 31 kD protein. Treatment of hepatocytes with ADP/Fe, which stimulates lipid peroxidation, led to induction of hsp 95, 80, 70, and 31 kD. In MH_1C_1 cells ADP/Fe only induced hsp 100, 85, and 70, but not hsp 80 and 31 kD. In human hepatoma cells ($HepG_2$) HNE or heat shock strongly induced synthesis of a hsp 70, the most conserved among the heat shock proteins.[159] In this cell line the hsp 70 was not inducible by ADP/Fe. It was convincingly shown that HNE acts on transcription; increased levels of hsp 70 mRNA were detectable by two different hybridization techniques. This result suggests that the induction of certain heat shock proteins (31 kD, 70 kD) occurs through a common trigger, which can be switched on either by heat shock, or by lipid peroxidation (ADP/Fe) or by HNE. It seems plausible that ADP/Fe is most effective in cells responding with a high rate of lipid peroxidation. In continuation of this work[119] it was shown that the common trigger probably is the so called heat shock factor (HSF). It is known that the activation of hsp synthesis is regulated by a specific sequence of DNA called heat shock element HSE, to which the HSF binds and thereby promotes transcription of the hsp genes. In experiments with HeLa cells[119] it has been demonstrated that HNE treatment (150 μM, 1 h) causes the binding of a specific protein, probably the HSF, to the DNA-HSE sequence regulating the expression of the hsp 70. Exactly the same effects were observed after exposure of cells to heat. These findings confirm that HNE is in fact able to act at the transcriptional level. Furthermore, the authors have concluded that their results show that "a product of lipid peroxidation rather than events occurring during the process or the release of active radical species is an adequate trigger of hsp synthesis." The exact mechanism by which HNE interacts with the HSF and promotes its binding to the HSE is not yet known and would deserve further investigations.

Reports on the effects of HNE on the expression of oncogenes also belong to the same line of research.[121] In erythroleukemic cells HNE at concentrations of between 0.1 and 10 μM caused a drastic reduction of the mRNA for c-myc oncogene. The reduction was transient and 6 h after treatment the c-myc mRNA level was restored to the control level. This suggests that HNE has the potential to achieve a rapid modulation of certain mRNA transcripts. The c-myc mRNA has a very short half life, and conclusive evidence whether HNE has inhibited its transcription or promoted its degradation has so far not been obtained.

MALONALDEHYDE

Structure

Aqueous solutions of MDA were first prepared by Claisen (160) through acid hydrolysis of β-ethoxy ac-

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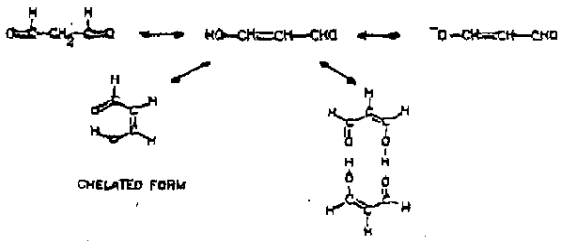

Fig. 13. Structures of MDA in aqueous solutions.

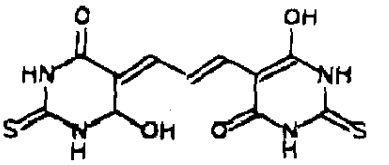

Fig. 15. Structures of the product formed by reaction of MDA with thiobarbituric acid.

rolein diethylacetal. Hüttel[161] crystallized the sodium salt of MDA which was said to be hygroscopic and can be stored only for a short time.[162] In aqueous solution MDA can exist in various forms depending on pH (Fig. 13). The pK value of the enolic OH group is 4.5.[162] At neutral and alkaline condition the predominant (more than 99%) form is therefore the enolate anion, whereas at acidic pH (pH < 4.5) MDA mainly exists in the tautomeric undissociated enol form as β-hydroxy acrolein and in equilibrium with the dicarbonyl form.[163] The configuration of the CC-double bond is mainly cis, and intramolecular hydrogen bonds favor the formation of a cyclic chelate form. Two cis-enols can also interact by intermolecular hydrogen bonds, and form a dimeric complex.[162]

For biological experiments aqueous solutions of MDA are most frequently prepared by acid hydrolysis (e.g., 1% H_2SO_4) at room temperature of commercially available bis-dimethyl- or bis-diethylacetal. In 1% H_2SO_4 MDA has a UV absorption maximum at 245 nm (ϵ = 13 700), in neutral or alkaline solutions the maximum is shifted to 267 nm (ϵ = 31 500). Dilute neutral or acidic solutions of MDA (below 25 μM) can be stored at 4 °C for 20 days without detectable change of the MDA content (162). With increasing concentration, however, aqueous MDA solutions become prone to aldol type self condensation leading to a dimer *31* and trimers *32* and *33* (Fig. 14),[164,380] which might be significant at room temperature and prolonged storage. At least five different condensation products were present in an MDA solution prepared by acid hydrolysis of the tetra-methylacetal in 0.33 M HCl at 60 °C and stored for 10 h at 4 °C.[165] These products could be separated by gel chromatography, and some of them showed fluorescence at 490 nm (Ex 365 to 395), which might interfere in fluorescence studies. In use of MDA for biological studies it should also be considered that MDA solutions prepared by acid hydrolysis of the bis-acetal may contain small amounts of β-ethoxy or β-methoxy acrolein,[166] which is a highly reactive aldehyde able to modify biomolecules much more readily than MDA itself. It has been reported that MDA solutions free of these contaminants can be prepared enzymatically by aldehyde dehydrogenase from 1,3-propanediol,[167] or from the chromatographically purified sodium salt of MDA, or by hydrolysis of *p*-nitrophenoxy acrolein.[168]

Determination

A great number of methods were introduced for determination of MDA in free and/or bound form. They include either reactions leading to colored or fluorescent products,[169–174] or chromatography such as TLC,[176–177] HPLC,[178–188,207,391] or GC[189–191,392] with or without derivatization. Among all, the most common method is the colorimetric or fluorimetric determination of MDA or MDA-like materials by the thiobarbituric acid (TBA) assay (for review see 8, 391). This assay was first used by food chemists to measure rancidity. Sinnhuber et al.[192] characterized the chromogen as condensation product of two molecules of TBA with 1 molecule of MDA (Fig. 15). The product has an absorption maximum at 532 nm (in acidic

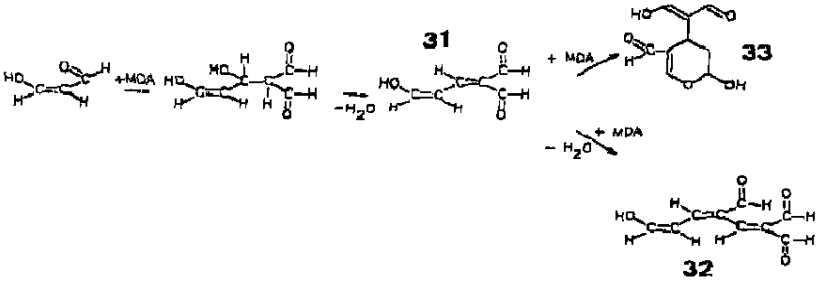

Fig. 14. Dimers and trimers of MDA which can be formed in aqueous solution.

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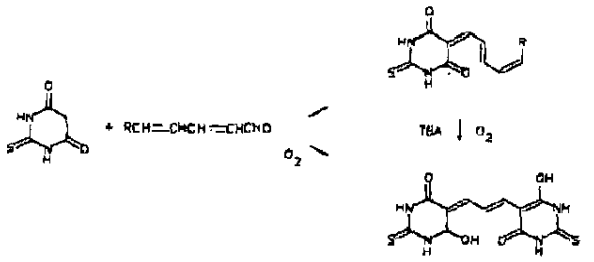

Fig. 16. Reaction of 2,4-alkadienals with thiobarbituric acid. In the presence of oxygen the same product is formed as with MDA.

conditions of the assay) with a very high molar absorptivity of 153,000. It can be extracted from the aqueous phase into butanol, which can be of importance if turbid samples must be analyzed. The fluorescence maximum is at 453nm.

Treatment of biological samples with TBA under appropriate conditions often results in the formation of pink-colored products, which absorb in the 500–550 nm range. The simplicity of the test, its recommended calibration with authentic MDA, and the similarity in the color with that of the TBA-MDA complex has probably misled many scientists to believe that what they measure with the TBA assay was in fact free MDA resulting from lipid peroxidation. It is, however, well documented that MDA or MDA-like substances can also be formed during the assay from various precursors produced during lipid peroxidation such as oxidized lipids, 2-alkenals, 2,4-alkadienals (Fig. 16), 4-hydroxyalkenals or MDA bound to proteins (193–198,202). Moreover, many substances which are not related to lipid peroxidation (e.g., some sugars and amino acids, urea, biliverdin, pharmaceuticals, glyoxal, furfuraldehyde) may also form a TBA complex with significant absorbance around 530–535 nm.[199–201] The absorbance at 532 nm by its own is therefore not indicative of MDA and it seems more appropriate to express the results in terms thiobarbituric acid reactive substances (TBARS) rather than MDA. On a molar basis the absorption produced by compounds other than MDA is usually low compared to MDA, thus the interference can be weak or strong depending on their relative ratio to MDA and the reaction conditions. If high concentrations of ferrous ions are present in the reaction mixture, the yield of 532 nm absorbing material can be significantly higher, for example treatment of hydroperoxides of arachidonic acid with TBA (10% TCA, 30 min at 100 °C) in the presence of 2.5 mM $FeSO_4$ gives a significant 532 nm absorption with a molar absorptivity of 12,400–34,100.[206] To avoid errors introduced by chromogens which have the absorption maximum not at 532 nm it is strongly recommended to record the spectrum in the 430–600 nm range and to correct for possible background absorption. The exact amount of the MDA-TBA complex can also be determined by HPLC.

Numerous variations of the TBA assay differing in sample pretreatment, acid concentration, heating time, absence or presence of antioxidants and/or catalytically active ferrous ions were introduced. One should keep in mind that the purpose of such modified assays is rather to obtain an index for lipid peroxidation than to measure MDA. In assays where the sample is first treated under mild conditions with TCA (one volume sample plus two volume cold 10% TCA) and the clear TCA soluble fraction is subjected to the TBA reaction, free MDA is measured primarily. In tissue homogenates, serum, and blood, TCA precipitates not only the proteins but also most of the lipid material, which is present in form of lipoproteins. In case of peroxidized microsomes the use of this method gave precisely the same values as found in parallel assay when MDA was determined by HPLC.[188] In assays where the whole acidified sample is heated with TBA for a longer time the resulting absorption at 532 nm can come from preexisting MDA, protein-bound MDA, lipid peroxides, and many other substances (e.g., aldehydes) which degrade to MDA in the hot acid.

Assays using phosphoric acid (1%) instead of TCA were reported to measure primarily protein-bound MDA (for a review see 8). The very commonly used assay developed by Yagi[204,205] was designed to measure lipid peroxides present in plasma or whole blood. Here the serum is first treated with phosphotungstic acid, which precipitates the protein and lipids (e.g., lipoproteins), and the protein/lipid pellet is then heated with TBA in acetic acid. Clearly, since the serum proteins are present, the assay would also determine potentially present protein-bound MDA. A method designed for microdetermination of lipid peroxides in oxidized lipids employs a modified TBA assay supplemented with 2.5 mM $FeSO_4$ (to promote degradation of preexisting peroxides) and BHT (to prevent formation of new peroxides during the assay).[206]

To improve the specificity, various HPLC methods for MDA determination were introduced. They can be divided into three categories, that is, precolumn derivatization,[180,182,185–187] postcolumn derivatization[183,199] and direct determination without derivatization.[179,182,184,188,207] Reagents employed for precolumn derivatization are TBA,[180] dansyl-hydrazine,[181,182] dinitrophenylhydrazine,[185,186] and methylamine + acetaldehyde.[187] Postcolumn derivatization can be made with the TBA reagent.[183,199] The direct HPLC determination takes advantage of the UV-absorption of MDA. Separations were described for ODS columns[184,188,207] and size exclusion columns.[182] Finally several GC and GC/MS

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methods were introduced. They require that the MDA is first derivatized to a volatile product. Proposed derivatives were the conversion to the tetra-methylacetal[191] and the derivatization with methylhydrazine,[190] 2-hydrazino-benzthiazole,[190] or penta-fluorophenylhydrazine.[392]

An investigator new in the field of lipid peroxidation is likely to be confused by the many methods which were introduced for determination of MDA or TBARS respectively. Which method should he use, are there good ones and others not so good? It all depends on the question to be answered. If one wants to know whether a certain treatment has induced lipid peroxidation (e.g., in cells or cell fractions) or whether a sample contains some remnants of lipid peroxidation, the first method of choice should be the TBA assay, preferably with the whole sample. We are not aware of any studies with true biological samples (exceptions might be, of course, pure model compounds as, for example, oxidized oleic acid), where TBA gave a negative result, but other methods indicated the existence of peroxidation. If the TBA assay is not positive it makes no sense to try one of the more sophisticated methods. However, if the TBA assay gives a positive result, additional analysis should be performed to prove that this is truly related to preexisting MDA or protein-bound MDA, or other lipid peroxidation products.

Occurrence

A huge number of investigations exist in which MDA or TBARS were measured in model systems and biological samples. We have compiled a representative list with the main emphasis on publications which appeared within the recent years (Table 6). Since oxidative stress and lipid peroxidation might be involved causally in the pathogenesis of human diseases, the determination of remnants of lipid peroxidation in human blood or serum has received considerable attention. Table 7 gives a survey on MDA or TBARS values which were reported to be present in human blood, serum, or plasma of healthy human subjects.[208–219] The values show, depending on the method of determination, an extremely wide variation from 0.0 to 47.2 nmol/mL plasma, which clearly indicates the problems inherent to all assays. With the most specific methods, MDA was either undetectable in plasma (HPLC without derivatization[207]) or low (0.6 nmol/mL with HPLC and TBA postcolumn derivatization).[199] The highest values of 35 to 47 nmol/mL were found in an assay where the whole plasma was heated for 30 min at 100 °C in strong acidic TBA reagent and only the developed 532 nm absorbance was measured.[215,218] The component(s) in plasma responsible for the formation of the chromogen in this assay as well as in the assays, where values in the medium range were obtained, is/are largely unknown and would deserve more attention, because there seems to be no doubt that increased values are often associated with human diseases. Thus increased plasma TBARS were reported to occur in patients with heart diseases[217] and diabetes[220] as well as in chronic smokers[221] and in elderly persons.[210]

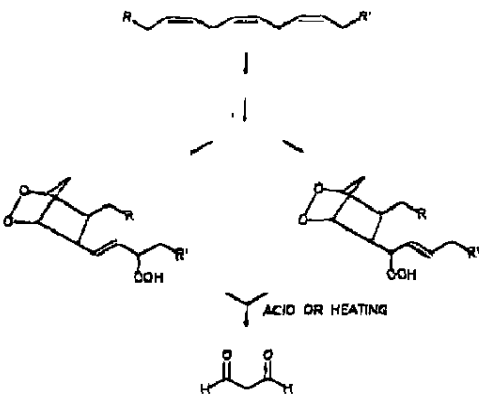

Fig. 17. Possible mechanism of formation of MDA from PUFAS according to Pryor and Stanley.[222]

Mechanism of formation

MDA in fresh or peroxidized biological samples results mainly from the oxidative degradation of PUFAS with more than two methylene-interrupted double bonds. In mammalian tissue the precursors for MDA will therefore be mainly the PUFAS arachidonic acid (20:4) and docosahexaenoic acid (22:6). Possible mechanisms for the formation of MDA from these PUFAS were proposed by Pryor and Stanley[222] (Fig. 17). It involves the formation of bicyclo-endoperoxides as intermediates, which subsequently break down to free MDA by thermal or acid-catalyzed reactions. Another mechanism was suggested by Wilson.[223] Here the intermediate is a peroxidized PUFA with a vicinal hydroxy–hydroperoxy grouping.

Frankel et al.[191] used an acetalation-acid decomposition method to investigate the formation of MDA from lipid oxidation products. A wide assortment of purified primary and secondary oxidation products from linoleate and linolenate (Fig. 18) were treated at ambient temperature with a mixture of methanol, trimethylorthoformate and anhydrous HCl for 18 h. The formed MDA-tetramethylacetal was then quantified by GC-MS. The highest yield (about 25 mol%) of MDA was obtained from the five-membered linoleate hydroperoxy epidioxide with an allylic trans-double bond. Rich sources of MDA (about 1 to 5.8 mol%) were also various hydroperoxy epidioxides, hydroperoxy bis-epi-

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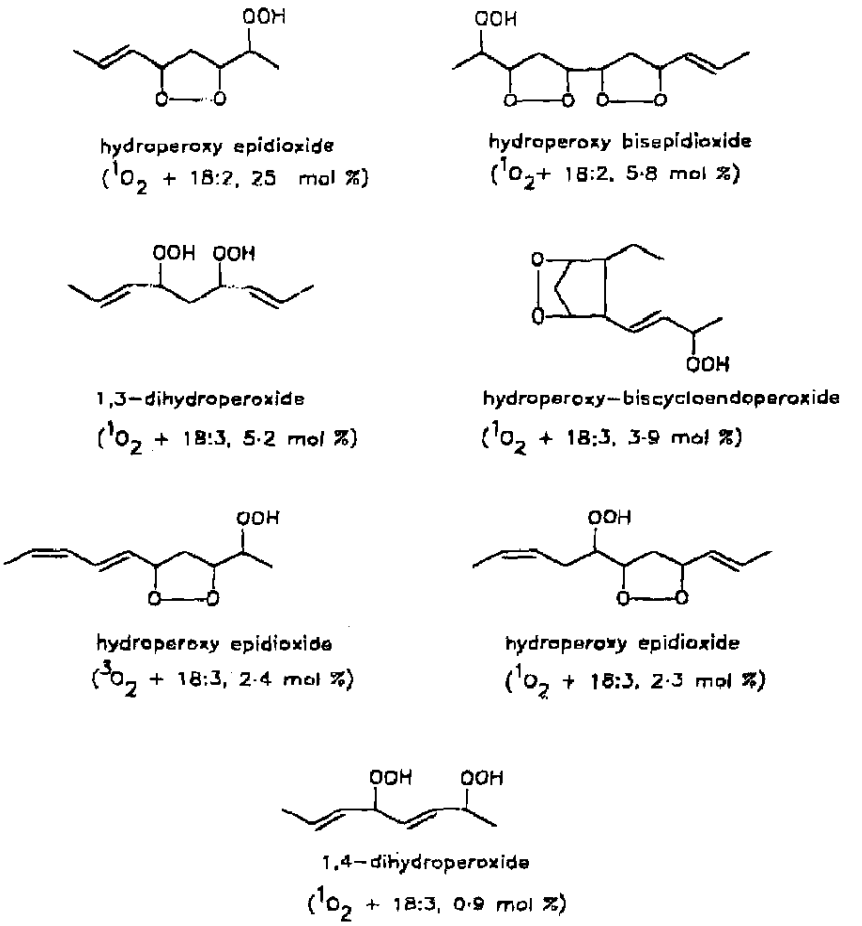

Fig. 18. Lipid oxidation products giving MDA-tetramethylacetal upon acetalation-acid decomposition (191). As indicated in brackets, the compounds were prepared by photooxidation with singlet oxygen ($^1O_2$) or autoxidation ($^3O_2$) from linoleate 18:2 or linolenate 18:3; given in brackets is also the yield of MDA in mol%.

dioxides, hydroperoxy bis-cycloendoperoxides, and 1,3- and 1,4-dihydroperoxides from linolenate (Fig. 18). Very little MDA (0.2 to 0.5 mol%) was given by monohydroperoxides of linoleate or linolenate. No MDA was formed from linoleate six-membered hydroperoxy epidioxides with an endocyclic double bond, and from lino- lenate 1,7- and 1,8-dihydroperoxides. These studies confirm the mechanism postulated by Pryor et al.[222] that in autoxidized lipids the main source of MDA will be five-membered hydroperoxy epidioxides and hydroperoxy bis-cycloendoperoxides derived from PUFAS with more than two double bonds. Note that linoleate hydroperoxy epidioxide, which appears to be the richest source of MDA, is a photooxidation product (singlet O_2) and likely not present in autoxidized lipids.

We still want to propose two other mechanisms for the formation of MDA from PUFAS with more than two double bonds. The principal steps in case of arachidonic acid are shown in Fig. 19. They involve a successive degradation (formation of hydroperoxides followed by β-cleavage) of the fatty acid chain to a hydroperoxyaldehyde, which could then yield MDA by β-scission or an acrolein radical, which can react with an OH-radical to MDA in the enol form (Fig. 19).

It is generally agreed that oleic acid or linoleic acid are weak precursors for MDA. We have found,[4,8] for example, 4.5, 4.9, 4.7, and 7.6 mol% free MDA (determined by HPLC) in iron/ascorbate autoxidized linolenic, gamma-linolenic, arachidonic, and docosahexaenoic acid, whereas from linoleic acid the yield was only 0.5 mol%, and no MDA was present in oxidized oleic acid. In these systems the TBARS-values were only slightly higher than free MDA which indicates that most of the MDA precursors in fact decomposed to free MDA already during the autoxidation period.[4]

In certain tissues MDA can also be formed by en-

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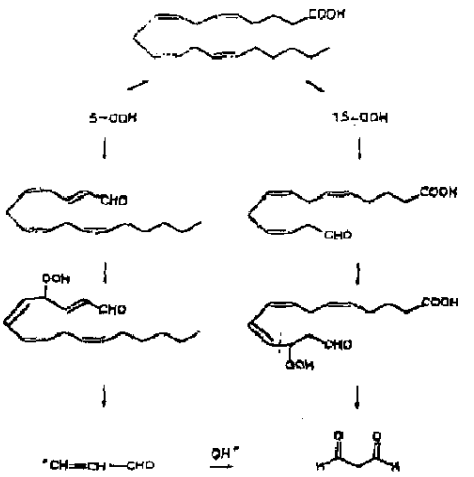

Fig. 19. Possible mechanisms of formation of MDA through autoxidation of intermediate polyunsaturated aldehydes derived from β-cleavage of 5-hydroperoxy or 15-hydroperoxy arachidonic acid.

zymatic processes.[176,224] MDA, thromboxane A_2 and the corresponding C_{17} compounds are formed, for example, in a ratio of 1:1:1 by human platelet thromboxane synthetase from PGH_2, PGH_1 and PGG_2. The PGH_1, 8-iso-PGH_2 and 13(S)-hydroxy-PGH_3 gave only MDA and the corresponding C_{17} compound, but not thromboxane A_2. The reaction mechanism for the formation of MDA from PGH_2, as proposed by Hecker and Ulrich,[224] is given in Fig. 20. Recently it was shown that MDA can also be formed enzymatically from spermine.[225] A polyamine oxidase converts spermine into 3-amino-propanal which is then oxidized by an aminoxidase to MDA. Formation of MDA by this enzymatic process was observed in homogenates of transformed kidney cells supplemented with spermine. Spermidine cannot be converted enzymatically to MDA.

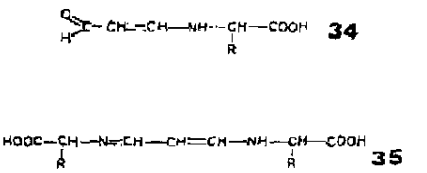

Fig. 21. Structure of the 1:1 and 1:2 condensation products of MDA with amino acids.

Model reactions

Among the three principal forms of MDA that can exist in aqueous solutions the enolate anion, which is the main species at pH 7.4, is of low reactivity. Under physiological conditions MDA is therefore not, as frequently said, a highly reactive compound, rather the contrary is the case. The reactivity increases at lower pH when the β-hydroxyacrolein becomes the predominant species. This form of MDA is an electrophile which can react with nucleophiles in a Michael type 1,4-addition reaction similarly to other α,β-unsaturated aldehydes (e.g., acrolein, HNE). Resonance stabilization of the intermediary formed anion is said to favor this type of reaction[226] which gives a β-substituted acrolein as end product. This primary 1:1 reaction product may then further react to give 1:2 adducts (Fig. 21).

Treatment of glycine with MDA under strong acidic conditions and at high concentrations gave a solid precipitate which was characterized as the 1:1 adduct enaminal, that is, *N*-propenal-amino-acetic acid *34*.[226] The yellow supernatant is fluorescent, and Chio and Tappel[227] isolated from it the 1:2 adduct *35*. Analogous 1:2 adducts with the general formula *35* were also formed under strong acidic conditions (1 N HCl) from Val, Val-methylester, and Leu. At strong acidic conditions (10 N HCl, 25 °C) the guanidino-group of Arg reacts with MDA to D-*N*-(2-pyrimidinyl)-L-ornithine.

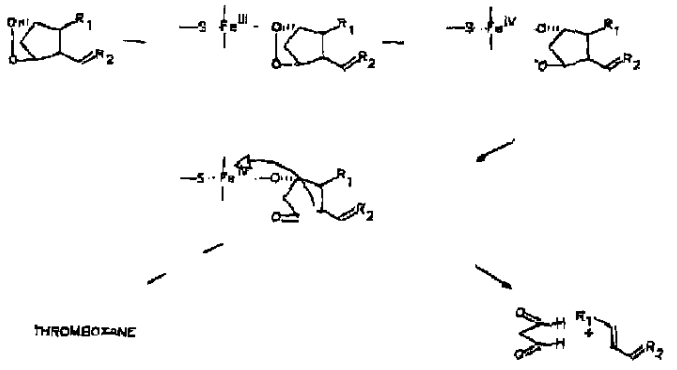

Fig. 20. Mechanism of formation of MDA from PGH_1 as proposed by Hecker and Ulrich.[224]

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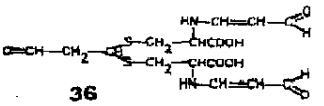

Fig. 22. Structure of the reaction product of MDA with cysteine. MDA:Cys 3:2.

This reaction was occasionally used to modify selectively Arg-residues in proteins.[229] In a careful study Nair et al.[230] investigated the reaction of MDA with several amino acids under conditions (pH 4.2, 20–200 mM) which are somewhat closer to the physiological situation. The formed products were characterized by NMR, elemental analysis, and UV- and mass-spectroscopy. His, Tyr, Try, and Arg reacted exclusively at the α-amino-group to the corresponding enaminals that is, the 1:1 adducts *34*, and under these mild conditions obviously no 1:2 adducts *35* were formed even with large excess of amino acids and extremely long reaction times of 13 days at room temperature. The methyl-esters of His, Arg, Try, and Lys also reacted with MDA to enaminals[228,230] of the general structure R–NH–CH=CH–CHO. The reaction of cysteine with MDA at neutral pH and room temperature leads to a product containing two cysteine residues and three molecules of MDA[231,232] (Fig. 22). We have studied the reaction of MDA (0.1 mM) with Gly and GSH (1 mM) at pH 7.4, and found virtually no reaction over 5 h (Fig. 23). In these experiments the MDA concentration was determined over time by HPLC. Under the same conditions cysteine (1 mM), serum albumin (1 mM), and serum (60 mg/mL) led to a consumption of 65, 50, and 70%

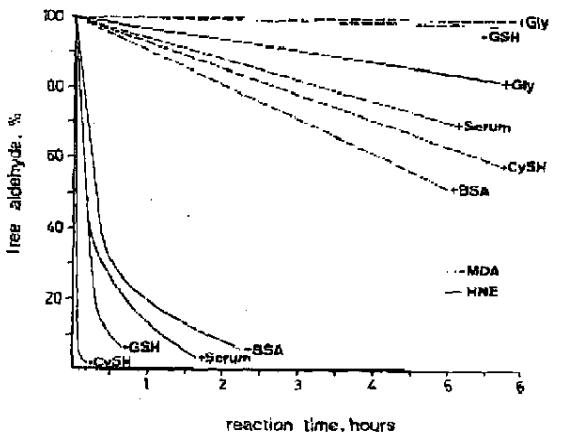

Fig. 23. Kinetics of the reaction of MDA (0.1 mM) or HNE (0.1 mM) with glutathione (GSH 1mM), cysteine (CySH 1mM), glycine (Gly 1 mM), bovine serume albumine (BSA 1mM) or human serum (60 mg/mL). The reaction was carried out at pH 7.4 and the consumption of MDA or HNE was followed by HPLC. (redrawn from Fig. 9 and 10 in ref. 2).

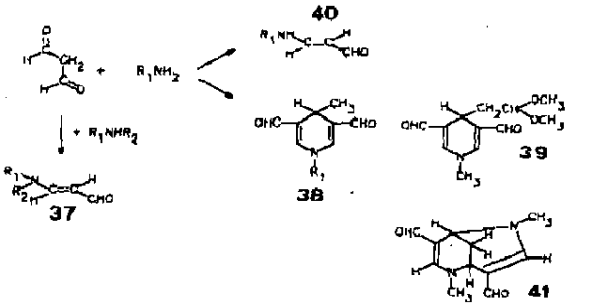

Fig. 24. Products identified in a reaction mixture of MDA with secondary or primary amines. Note that *39* and *41* result from impurities in the MDA solution.

of MDA within 5 h. HNE, on the other hand, was much more reactive and was fully consumed (with Cys, GSH, serum, BSA) within 1 h or less. This experiment clearly indicates that under physiological conditions MDA is considerably less reactive than α,β-unsaturated aldehydes and its reaction with the α-amino-group of amino acids are in fact not favored.

The reaction of methylamine and several secondary amines with MDA (prepared by acid-hydrolysis of tetramethoxy-propane) at neutral or weak acidic pH and 37 °C was studied by Kikugawa et al.[234–237] Secondary amines (dimethylamine, diethyl-amine, piperidine, pyrrolidine, and morpholine) gave the 1:1 adduct, that is, β-dialkylamino-acrolein[236] (Fig. 24,*37*). These products are unstable and again gave a pink chromogen in the TBA assay. The reaction with methylamine was much more complex and 4 products could be isolated and identified: the fluorescent compounds 1,4-dimethyl-1,4-dihydro-pyridine-3,5-dialdehyde *38* and 1-methyl-4-(dimethoxymethyl)-1,4-dihydropyridine-3,5-dicarbaldehyde *39*, and the nonfluorescent compounds β-methylamino-acrolein *40* and di-azo-bicyclo[3.3.1.]-3,7-nonadien-di-aldehyde *41*[234] (Fig. 24).

Compound *39* and possibly compound *41* result from impurities (methoxy-acrolein, 3,3-dimethoxy-propionaldehyde) of the MDA solution used in these experiments. When the purified sodium salt of MDA was used instead of acid hydrolysates of tetra-methoxy-propane, methylamine gave only compounds *38* and *40*. The formation of compound *39* is thought to occur by the "Hantzsch dihydro-pyridine synthesis" from 1 mol methylamine + 2 mol MDA + 1 mol dimethoxy-propionaldehyde.[235] Similarly compound *38* is thought to be formed by condensation of 1 mol methylamine + 2 mol MDA + 1 mol acetaldehyde (Fig. 24), whereby acetaldehyde is formed in the reaction mixture from MDA by an unknown mechanism (a possibility is oxidation to malonic acid semialdehyde, followed by decarboxylation). The formation of dihydro-pyridine

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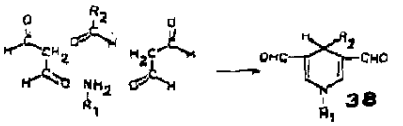

Fig. 25. Mechanism of the formation of a dihydropyridine derivative by reaction of MDA and a saturated aldehyde with a primary amine. The stoichiometry is 2:1:1.

derivatives with the structure *38* in reactions of primary amino groups with MDA alone or MDA in mixture with saturated aldehydes is now well established, and mechanisms of the reaction have been proposed[235,238] (Fig. 25). Since during lipid peroxidation MDA will always be formed together with other aldehydes including saturated aldehydes, such as hexanal, it is reasonable to assume that dihydro-pyridine derivatives can also be formed during lipid peroxidation in biological systems.

Compared with free amino acids, proteins are much more readily modified by MDA under physiological conditions.[227,239-242,246] It has been suggested that the amino acid residues in a protein provide a more favorable environment for reactions with MDA. It has also been suspected that modification of proteins might be caused not by MDA itself, but by some of its condensation products.[230] In polylysine, MDA led to three types of modifications of the ε-amino-groups (243). Twenty-two percent of the incorporated MDA was present as instable amino-propenal *42*, 77% were thought to have formed the more stable crosslinks with the amino-imino-propen structure *43*, and less than 1% had the dihydro-pyridine structure, that is, 1,4-dihydro-pyridine-3,5-dicarbaldehyde *44* (Fig. 26). Similar results were obtained with BSA modified with MDA (10

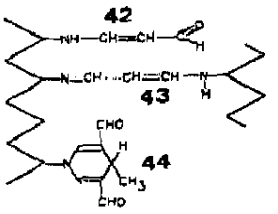

Fig. 26. Modification of ε-amino groups in proteins by MDA.

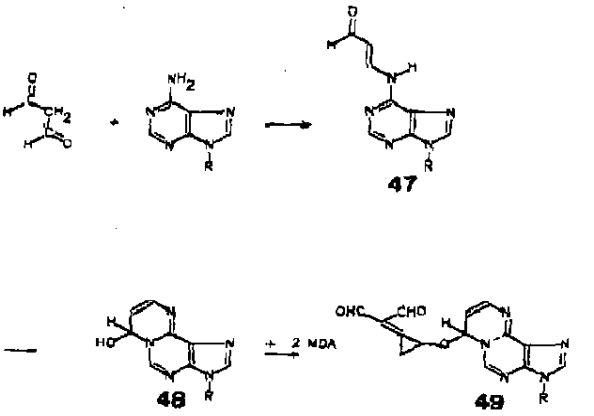

Fig. 28. Reaction of adenosine with MDA gives via *47* the 1:1 adduct AM_1 *48* and the 1:3 adduct AM_3 *49*.

mM) at 37 °C for 24 h. Here, MDA modified 26 lysine residues out of the total 56 present in this protein. In 11 residues MDA was bound to the ε-amino-groups as amino-propenal and 0.3% residues formed the 1,4-dihydro-pyridine structure. The remaining modifications are probably intra- and intermolecular cross-links with the amino-imino-propen structure.[244] That MDA can introduce cross-links in proteins has also been shown in various other studies, and it is generally agreed that they also have the amino-imino-propen structure, although this assumption is based only on indirect evidence. Amino acid analysis of MDA-modified proteins indicates that besides lysine residues His, Tyr, Arg, and Met may also be altered to some extent.[241]

Reaction of MDA with nucleosides is another subject which has received considerable attention[245-250] because of its mutagenicity. Deoxy-guanosine is the most reactive base and with MDA forms a 1:1 adduct GM_1 *45*[245] and a 1:2 adduct GM_2 *46*[247,248] (Fig. 27). Adenosine gives a 1:1 adduct AM_1 *47*[246,249] and a 1:3 adduct AM_3 *49* (Fig. 28). Cytidine gives a 1:3 adduct CM_3 (Fig. 29),[249] and no reaction was observed with thymidine. The reaction of MDA with nucleosides is strongly accelerated by decreasing pH. With guanosine, the reaction proceeds 18 times faster at pH 1.0 than at pH 4.2. Furthermore, rather long reaction times and high concentrations of the reactants are neccessary

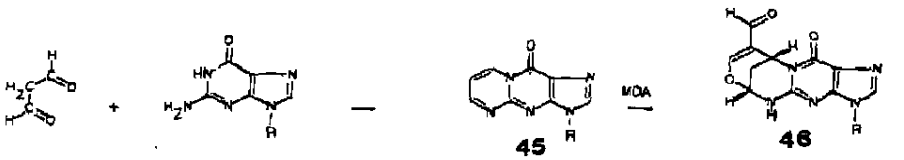

Fig. 27. Reaction of deoxyguanosine with MDA gives the 1:1 adduct GM_1 *45* and the 1:2 adduct GM_2 *46*.

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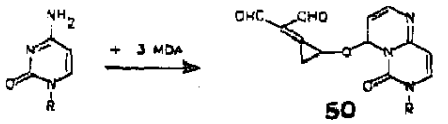

Fig. 29. Reaction of cytidine with MDA gives the 1:3 adduct CM_3 50.

to obtain modifications of nucleosides, which makes it rather unlikely that such modifications could occur to a large extent in vivo. The 1:1 adduct of deoxyguanosine with MDA, that is, 3-(2 deoxy-β-D-erythro-pentafuranosyl)-pyrimido [1,2-α]-purine-10(H)-one *45* was the only product found in MDA-treated DNA after enzymatic digestion.[252] After alkaline hydrolysis of MDA-treated RNA or DNA no modified bases were found, probably due to their instability under alkaline conditions. In RNA, however, the content of CMP, GMP and AMP was decreased which suggests that a part of them was altered by MDA.[250] It has been proposed that MDA can modify double-stranded DNA by formation of amino-imino-propen crosslinks between the NH_2 groups of a guanosine base and the NH_2 group of the complementary cytosine base (Fig. 30).

Metabolism of MDA and excretion of MDA derivatives in urine

In rat liver MDA can be oxidatively metabolized to CO_2 and H_2O. In this process mitochondrial aldehyde dehydrogenases first convert MDA to malonic acid semialdehyde, which spontaneously decarboxylates to acetaldehyde. Acetaldehyde is then further oxidized by aldehyde dehydrogenases to acetate, and further to CO_2 and H_2O.[253] In mouse liver the formation of malonic acid semialdehyde is catalyzed by a cytosolic aldehyde dehydrogenase. A minor fraction of MDA can also be oxidized in liver to malonate, which is converted to malonyl-CoA and decarboxylated to acetyl-CoA.[254]

In the urine of rats and humans various enaminals (RNH–CH=CH–CHO) were found arising from metabolic degradation of MDA-modified proteins (Fig. 31). They are N^{ε}-(2-propenal)lysine *52*,[255–257] N^{α}-acetyl-N^{ε}-(2-propenal)lysine *53*,[258] *N*-(2-propenal)-serine *55*,[259] and *N*-(2-propenal)etholamine *54*.[260] The greater part of these products is not of endogenous origin, but results from degradation of MDA-modified proteins taken in with food of animal origin.[257] An increase of these MDA-amino acid conjugates in urine was observed in rats under vitamin E-deficient conditions,[49,261] after CCl_4-poisoning,[261] after injection of iron-nitriloacetate (which stimulates lipid peroxidation,[262] or in rats fed a cod liver oil diet followed by a 24-h period of fasting.[261]

The MDA level in normal human urine was recently reported to be in the range of 0.2 to 0.8 μM, determined as pentafluorphenyl-hydrazine derivative by GC with electron capture detection.[392]

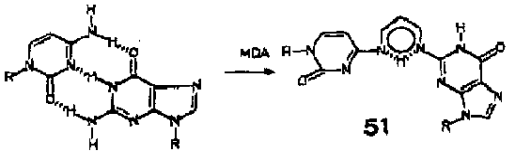

Fig. 30. Possible MDA cross-links between guanine and cytosine bases in double-stranded DNA.

ACROLEIN

Occurrence and reactivity

Acrolein $CH_2=CH–CHO$ is a widely used bulk chemical and occurs as an ubiquitous pollutant in the environment, because it is formed during incomplete combustion of petrol, coal, wood, and plastic material (for review see 291, 292). Acrolein is also present in the gas phase of cigarette smoke in amounts of about 25 to 140 μg/cigarette.[12] It has been known for a long time that acrolein is formed in overheating frying oils, and the name "acrolein" derives from the acrid smell of overheated oils. Moreover, acrolein is a toxic metabolite formed in bio-transformation of allyl alcohol,[11,293] allylamine,[294] and allyl-formate,[295] and the widely used anticancer drug cyclophosphamide.[291,296–298] Acrolein may also be formed intracellularly by enzymatic oxidation of polyamine metabolites.[299,300] Small amounts of acrolein can also be formed from PUFAS during lipid peroxidation. Other 2-alkenals (for review see 4) identified in peroxidized microsomes (ADP/Fe) are 2-pentenal, 2-heptenal, 2-octenal, and 2-nonenal. Autoxidation of fatty acids under mild conditions also leads to a formation of a number of 2-alkenals with chain length from 3 to 11 carbon atoms. The literature on the occurrence of ac-

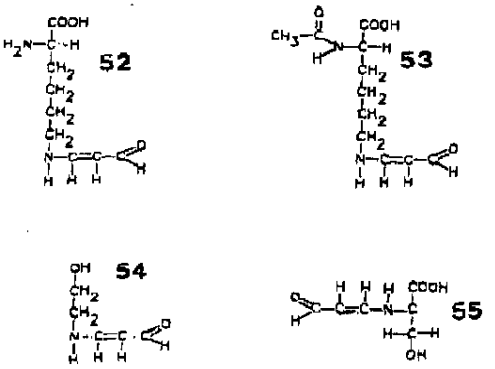

Fig. 31. MDA metabolites present in urine.

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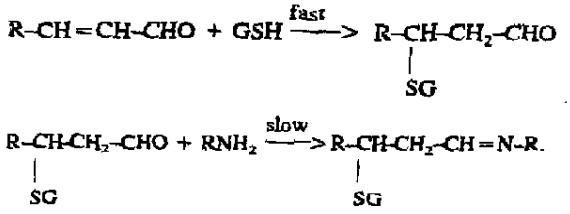
rolein and its hazardous effects has been reviewed several times (see, e.g., refs.12, 291, 292), and we therefore limit this chapter to a few findings, which are, at least in part, of interest in connection with the previous chapters on HNE and MDA.

Among all α,β-unsaturated aldehydes including 4-hydroxyalkenals, acrolein is by far the strongest electrophile and therefore shows the highest reactivity with nucleophiles such as the thiol- or amino group.[1,12] It can be seen from Table 3 that acrolein reacts about 110–150 times faster with GSH than crotonal or HNE.[70] The mechanism of the reaction of acrolein, crotonal, and higher 2-alkenals with GSH is principally the same as described for HNE (Fig. 5A). However, the 1:1 adducts *18* do not cyclize and therefore have the free aldehyde function, which could undergo secondary reaction, such as, for example, Schiff's base formation.



The same reaction probably takes place with SH groups in proteins. With cysteine the 1:1 adduct is only an intermediate product which rapidly reacts with a second molecule of cysteine to the thiazolidine derivative *24* (Fig. 5C), which is the only product in reactions of 2-alkenals with cysteine.[71] The thiol adducts of acrolein are considerably more stable than the adducts formed by all other α,β-unsaturated aldehydes (Table 3). The dissociation constant of the acrolein adducts are about 10 to 10,000 times lower than those for the other aldehydes.[70]

The reaction of acrolein with amino groups (RNH_2) is much slower than those with the thiol group[12] and results in β-substituted propanals ($R–NH–CH_2–CH_2–CHO$) and Schiff's bases ($R–NH–CH_2–CH_2–CH=N–R$). In proteins, acrolein preferentially attacks free SH groups of cysteine residues, ε-amino groups of lysine residues and histidine residues. In α-1-proteinase of plasma, acrolein modified lysine and histidine, but not arginine or the other amino acid residues (301). The structure of the lysine- or histidine-acrolein conjugates was not determined. Treatment of adult human hemoglobin with acrolein lead to a heterogenous mixture of modified hemoglobin molecules including cross-linked dimers.[302]

The reaction of acrolein and crotonal with nucleic acid bases has received considerable attention (Fig. 32). Acrolein reacts at neutral pH with guanosine[303] yielding cyclic 1:1 propano-deoxy-guanosine adducts *56,57*. The proposed mechanism leading to adduct *56* involves Michael addition of the N-1 amino group of deoxy-guanosine to carbon 3 of acrolein followed by ring closure between N-2 of deoxy-guanosine and the C_1 of acrolein. Adduct *57* is formed by ring closure in the opposite direction. Crotonal forms with deoxy-guanosine only the 1:1 cyclic propano-deoxy-guanosine adduct[304] with the structure *58*. The cyclic 1:1 guanosine adducts *57* and *58* were also formed when calf thymus DNA was reacted with acrolein or crotonal at pH 7.0, 37 °C. The number of modified guanosine residues was considerably higher in acrolein-treated DNA.[303] Cyclic adducts are also formed by reaction of acrolein with cytosine and adenine derivatives.[394] Application of an immunoassay for acrolein deoxyguanosine adducts in DNA from *Salmonella typhimurium* exposed to acrolein revealed that cyclic 1,N^2-propano-deoxyguanosine adducts are in fact formed in vivo in a dose dependent manner.[395] The maximum modification with 13 mM acrolein was 5 μmol adduct per mol deoxyguanosine.

Crotonal is also one of the metabolites formed in rat liver from the cyclic nitrosamine *N*-nitrosopyrrolidine

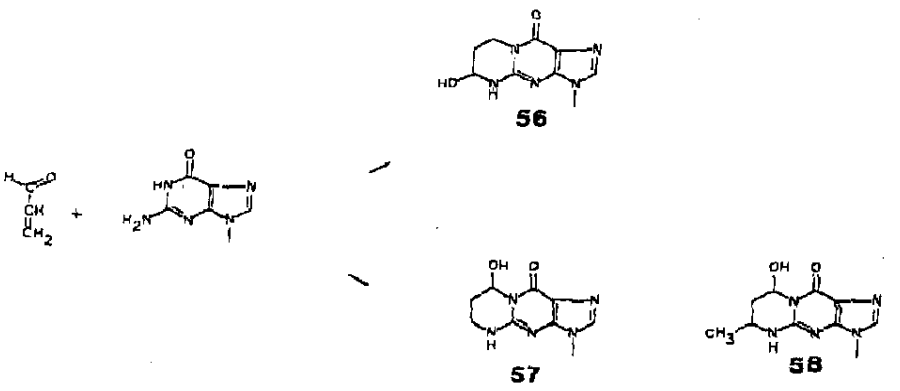


Fig. 32. Reaction of acrolein with guanosine gives the 1:1 adducts *56* and *57*. Crotonal gives with guanosine an analogous adduct *58*.

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(NPYR). NPYR is a hepatocarcinogen and considered as a prototype for environmentally important nitrosamines. With a modified ^{32}P-postlabeling method the cyclic 1-N^2-propanodeoxyguanosine adduct *58* was detected in hepatic DNA of rats treated with NPYR, the crotonal adduct *58* was also present in skin DNA of mice treated topically with crotonaldehyde. The metabolism of NPYR occurs via an α-hydroxylated, unstable intermediate which spontaneously decomposes to crotonaldehyde. The hydroxylated intermediate can also be generated in vitro from α-acetoxy-*N*-nitrosopyrrolidine. Calf thymus DNA reacted with α-acetoxy-*N*-nitrosopyrrolidine in the presence of an esterase also contained modified guanosine bases including the propanodeoxyguanosine adduct *58*.[397,398]

Cytotoxicity and inhibition of proliferation

Acrolein is highly cytotoxic towards mammalian cells. Human bronchial epithelial cells exposed to acrolein for 1 hour in a thiol-free (i.e., without calf serum) medium were severely damaged if the concentration exceeded 10 μM. The LD_{50} determined by trypan-blue in these cells was about 20 μM acrolein.[305] Colony forming efficiency of these cells was significantly decreased already at a 10-fold lower concentration of about 1 μM. This dose is comparable to the dose which reduces colony forming efficiency in cultured human bronchial fibroblasts (EC = 3 μM)[97] or in normal human fibroblasts (EC = 0.66 μM)[306] to 50%. Interestingly, fibroblasts with a defect in the DNA repair system (derived from patients with Xeroderma pigmentosum) were more sensitive towards acrolein, a 50% reduction of colony-forming efficiency was already obtained at about 0.15 μM acrolein.[306] This suggests that a decrease of colony forming efficiency is caused by DNA damage. In rat whole embryo cultures the EC_{50} for malformation and embryolethality are 2.8 μM and 8.3 μM, respectively, if assayed in a serum free medium.[307] The embryotoxicity of acrolein was about 10 fold lower in culture media supplemented with serum.[307,308] This influence of the components of the medium on toxicity was observed with many other α,β-unsaturated aldehydes and other cell lines (see, e.g., 21). It can easily be explained by the fact that in the presence of serum a part of the aldehyde will always be scavenged and thereby detoxified by SH groups of the serum proteins.

A comparison of the cytotoxicity data for acrolein with those of the higher homologs indicates that the cytotoxicity sharply decreases from acrolein to crotonal, and then increases again with the increasing chain length of the aldehyde. For example, in *Salmonella typhimurium* TA 104 the maximum nontoxic doses were[130] 0.9, 3.0, 0.9, 0.8, and 0.007 μmol per plate for acrolein, crotonal, 2-hexenal, 2-octenal, and 2-nonenal, respectively; for cultured fibroblasts[21] (Fig. 10) or umbilical vein endothelial cells[100] the cytotoxicity and also the effect on growth capability increased from 2-hexenal to 2-nonenal; acrolein was not used in these studies, but one may assume that it would have been more toxic than 2-hexenal. It is generally agreed that saturated aldehydes have a much lower toxicity than the 2-alkenals. In bronchial epithelial cells formaldehyde and acetaldehyde were toxic only at 200 to 5000-fold higher concentrations as compared to acrolein.[309] In human bronchial fibroblasts 100 to 300-fold higher doses of formaldehyde or acetaldehyde were required to reproduce the effects of acrolein.[97]

The exact mechanism by which acrolein and other 2-alkenals lead to cell death or inhibit cell proliferation are largely unknown, but it is reasonable to assume that what has been said about HNE is applicable here, too. In common with HNE, acrolein causes rapid depletion of thiols. This has been shown for human bronchial fibroblasts,[97] bronchial epithelial cells,[305] isolated hepatocytes,[310–314] and nasal mucosa of acrolein exposed rats,[315] and also for other systems. Exposure of cells to acrolein leads first, in a concentration-dependent manner, to a loss of cellular glutathione. When the GSH-level is decreased to a certain threshold level, protein thiol groups also become progressively modified.[305] In bronchial epithelial cells, for example, 3 to 10 μM acrolein causes a marked reduction of GSH, but has no effect on protein-SH. A substantial loss of proteinSH groups was observed at or above 10 μM acrolein.[305] It appears that cytotoxicity is dependent on glutathione depletion, whereas acrolein-induced growth inhibition occurs before a significant loss of GSH. GSH also protects against the embryotoxic and teratogenic effects of acrolein.[307,308] In acrolein-exposed bronchial epithelial cells, no increase in oxidized glutathione GSSG was observed, indicating that GSH was consumed by conjugation to acrolein, but not by oxidative stress.[305]

Acrolein can inhibit, as does HNE or other alkylating agents, enzymes with functional SH groups, for example, DNA-polymerase α (see ref. 97), the DNA repair enzyme O^6-methylguanine-DNA-methyl transferase or DNA-methylase.[316] In cells, such an inhibition only occurs when GSH is depleted. In isolated enzymes the inhibition is prevented by GSH. Cox et al.,[316] for example, studied the effect of acrolein on DNA-methylase from the urothelium and liver of rats in connection with cyclophosphamide-induced bladder cancer, and found the enzyme being inhibited at 10 μM acrolein to about 30 to 50%. This inhibition could be prevented by di-thiothreitol or GSH. Interestingly, these authors also found that acrolein-treated DNA loses its ability to act as substrate for DNA-methylase. Acrolein can

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therefore influence DNA methylation by inhibiting the enzyme or by modifying DNA, probably by covalent binding to guanosine residues.

Hepatotoxicity of acrolein and allyl alcohol

Allyl alcohol is a strong hepatotoxin which causes extensive periportal necrosis in the rodent liver (for review see 11). Several studies have demonstrated that the hepatotoxicity depends on the metabolism of the alcohol to acrolein by hepatic cytosolic alcohol dehydrogenases.[317,318] Allyl alcohol is therefore a source for in situ generation of acrolein in liver or hepatocytes, and consequently allyl alcohol administered to rats causes a rapid loss of liver GSH by its conversion to the 1:1 conjugate $GS-CH_2-CH_2-CHO$.[11,121,312,313,319] This spontaneous reaction has a very high reaction rate constant (see Table 3), and glutathione transferases are probably of minor importance for catalyzing the conjugate formation. In hepatocytes the aldehyde group of the conjugate is rapidly oxidized via an NAD^+-dependent reaction to $GS-CH_2-CH_2-COOH$.[312] In vivo, this product is then further metabolized to mercapturic acid. The urine of acrolein-treated mice contained *S*-carboxyethyl-mercapturic acid and *S*-carboxyethyl-mercapturic acid methylester.[320] In liver, a part of acrolein is also detoxified by aldehyde dehydrogenases forming acrylic acid $CH_2=CH-COOH$, which has only a low toxicity.[293] Allylic acid administration to animals does not reproduce the hepatotoxic effects of allyl alcohol/acrolein.[295,321] Consistent with that is the observation, that the hepatotoxic effect of allyl alcohol is increased in rats pretreated with aldehyde dehydrogenase inhibitors.[322,323] Inhibition of aldehyde dehydrogenase by cyanamide makes a subtoxic dose of allyl alcohol highly toxic.[323] On the other hand, induction of liver aldehyde-dehydrogenases by phenobarbital prevents allyl alcohol toxicity, GSH depletion, and lipid peroxidation.

Comporti et al.[11] consider allyl alcohol as a prototype of agents lowering liver GSH. If GSH reaches a certain threshold level, protein SH groups are also attacked and thereafter an abrupt onset of lipid peroxidation occurs in the liver.[11,323,324] Allyl alcohol-treated animals[323] exhale ethane, have significantly increased liver TBARS and a loss of polyunsaturated fatty acids in the liver lipids. Other effects observed[323] are an almost complete loss of cytosolic liver alcohol dehydrogenase, a destruction (about 50%) of cytochrome P_{450}, and accumulation of the lipid peroxidation product HNE[55] in the liver. Several findings suggest that besides GSH depletion, lipid peroxidation plays a causative role in the allyl alcohol/acroleinmediated liver damage and death of hepatocytes (for review see 11). Firstly, depletion of liver GSH by phorone or diethylmaleate caused only a marginally enhanced lipid peroxidation and no liver damage, indicating that glutathione depletion is not necessarily lethal. Second, the iron chelator desferrioxamine does not affect loss of GSH, but prevents allyl alcohol-induced lipid peroxidation and liver damage; this also indicates that mobilized iron ions are critical for the toxicity of allyl alcohol. All this suggests that allyl alcohol/acrolein stimulates lipid peroxidation in vivo in the liver by destruction of a GSH-dependent protective system. Similar effects have been reported for HNE which, in isolated microsomes, also stimulates lipid peroxidation probably by inactivating a membrane-bound enzyme which catalyzes the reduction of vitamin E radicals by GSH.[132,156] However, acrolein appears to act by a different mechanism since it failed to induce lipid peroxidation in mouse liver microsomes.

Toxic in vivo effects

Acrolein is toxic and severely irritating to the mucosa. Its vapor causes strong eye and nasal irritation. Inhalation of acrolein inhibits mucociliary transport and causes dysfunction, hyperplasia, and squamous metaplasia in the respiratory tract of laboratory animals.[291,292] Direct contact of liquid acrolein with eye or skin results in severe burns. Ingestion leads to acute gastrointestinal distress with pulmonary congestion and edema. Some acute toxicity data collected in Ullmann's Encyclopedia of Industrial Chemistry[325] are: LD_{50} (rat oral) 46 mg/kg, LC_{50} (mouse, inhalation) 165 mg/m^3 over 6 h, LD_{50} (rabbit, dermal) 562 mg/kg.

Acrolein can be detected by its odor at levels above 0.35 ppm; the lowest lacrimatory level is 0.5 ppm. A level of 5.5 ppm results in painful eye and nose irritation after 20 s, and 22 ppm in air is immediately intolerable.[325] In general, acrolein is unlikely to cause acute intoxication in humans because of the highly irritating smell and the lacrimatory effect. However, danger arises from accidental inhalation of acrolein in concentrations exceeding the toxic or lethal threshold. In one fatal accident, the concentration was 153 ppm and the time of exposure 10 min.[325]

Crotonal is much less toxic than acrolein.[326] It is perceptible at concentrations of 0.2 ppm (0.6 mg/m^3). After exposure to 15 ppm for 30 s, no symptoms of eye irritation occur, but 45 ppm cause clear irritation. The mucous membranes of the respiratory system are irritated at concentrations of 4.1 ppm. Because of its warning symptoms no acute intoxication in humans has occurred and no case reports have been published. For experiments with animals, the following data on acute toxicity are given in Ullmann's Encyclopedia of Industrial Chemistry,:[326] LD_{50} (rat, oral) 300 mg/kg, LC_{50}

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(rat, inhalation, 30 min) 4000 mg/m^3; LD_{100} (rat, subcutaneous) 180 mg/kg; LD_{50} (rabbit, dermal) 380 mg/kg. Symptoms of acute intoxication in animals are dyspnea, excitation, hemorrhagic rhinitis, and congested lungs with hemorrhagic edema.

ALDEHYDES AND FLUORESCENT LIPID PEROXIDATION PRODUCTS

Lipid peroxidation in biological samples is accompanied by the formation of fluorescent proteins and lipids which have emission maxima in the range of 430–470 nm (for review see 87, 88, 251). It has been proposed that these fluorescent substances are formed by condensation of MDA with amino groups in phospholipids or proteins to fluorescent Schiff's bases with a general 1-amino-3-imino-propen structure R–N=CH–CH=CH–NH–R'. This assumption was mainly based on numerous model studies performed with compounds bearing primary amino groups such as alkylamines,[234,235] amino acids,[87,226,230,246,251] amino acid methylesters,[228] phosphatidylethanolamine,[251] polylysine,[243] proteins,[239–242] and DNA.[250,251] The NH_2 group reacts with MDA under appropriate (strongly acidic) conditions to 1:2 adducts with the amino-imino-propen crosslink (Figs. 21, 26). These products have emission maxima in the 450–470 nm region and excitation maxima in the 360–400 nm region (for review see 87, 251). The similarity with the fluorescence of lipofuscin and ceroid pigments has further led to the assumption that these fluorescent products, which can be found in form of granula in many tissues and cells, also result from condensation of MDA with molecules bearing NH_2 groups. Lipofuscin extracted from brain or heart shows an emission maximum at 470 nm and an excitation maximum at 365 nm,[87,88] which is indeed very similar to the fluorescence reported for conjugated Schiff's bases. On the other hand, all investigators who have studied fluorescence of in vitro peroxidized microsomes, mitochondria, lipoproteins, and various tissues, reported that the excitation maximum of the fluorescent compounds formed (lipids, proteins) was at 360 nm and the emission maximum was at about 430 nm, which is significantly different from the fluorescent maxima of conjugated Schiff's bases and rather suggests that the fluorescence is due to chromophors of another structure.[87] The reaction of peroxidized arachidonic acid or docosahexaenoic acid with phosphatidylethanolamine gave a fluorophor with E_X/E_M at 360/430–440 nm, whereas MDA with the same phospholipid gave a fluorescence at 400/475 nm.[327] Treatment of erythrocyte membranes with lipid hydroperoxides[328] or xanthine/xanthine oxidase[329] also led to fluorescent products (E_X/E_M 375/438 nm and 390/450 nm, respectively) which had spectral properties clearly different from those produced by MDA-treatment (E_X/E_M = 398/467 nm). In vitro peroxidation (ascorbate/iron) of rat liver liposomes supplemented with glycine produced a fluorescence (E_X/E_M 360/430 nm) different from the conjugated glycine MDA Schiff's base (E_X/E_M 360/458 nm).[330] It was assumed that not MDA but relatively high molecular weight aldehydes contribute to the formation of 430 nm fluorescent substances. In oxidized low density lipoprotein the apolipoprotein B shows a strong fluorescence at E_X/E_M 360/430 nm, but again it is unlikely that it is due to MDA since fluorescence of MDA-treated LDL is at E_X/E_M of 390/460 nm.[331,332]

In numerous studies Kikugawa's group[87,234–237,243,250,328] has shown that MDA can form under physiological conditions with primary amines fluorescent dihydro-pyridine derivatives, and that monofunctional aldehydes, which are also formed during lipid peroxidation, can participate in and stimulate the formation of this fluorescent substance (Fig. 24–26). Reaction of methylamine (10 mM) with MDA (20 mM) at pH 7.0 (37 °C, 24 h) gave the nonfluorescent 1:1 adduct CH_3NH–CH=CH–CHO (*40*, Fig. 24) and the fluorescent 1,4-dimethyl-1,4-dihydro-pyridine-3,5-di-carbaldehyde *38*. No 1:2 adduct with the conjugated Schiff's base structure was formed. In 0.1 M phosphate buffer pH 7 the compound *38* has an E_X/E_M of 403/462 nm, while in chloroform the peak maxima for excitation and emission are at 390/446 nm. The formation of *38* was assumed to proceed according to the mechanism of the Hantzsch dihydro-pyridine synthesis and requires a 1-carbon unit to be eliminated. Fluorescent products analogous to *38* (Fig. 24) were also formed in reactions of MDA at pH 7 with 1-hexylamine, glycine, glycine-ethylester, ethanolamine, and 3-amino-propanol. Based on the fluorescence of MDA-treated polylysine (398/470 nm), hemoglobin A (390/460 nm) or erythrocyte ghosts, it was assumed that the main fluorophor is again a dihydro-pyridine formed by reaction with ε-amino groups of lysine residues.

Of particular interest is the observation that the formation of dihydro-pyridine derivatives occurs more readily in the presence of monofunctional aldehydes (acetaldehyde, propanal, butanal, hexanal, heptanal, benzaldehyde). The optimal ratio of primary amine, MDA, and aldehyde is 1:2:1–2, at which the dihydro-pyridine is produced almost quantitatively. Since alkanals are formed together with MDA during lipid peroxidation it is reasonable that similar reactions can occur during in vivo and in vitro lipid peroxidation.

It has also been demonstrated that compounds with a fluorescence maximum around 430 nm are formed in reactions of aldehydes other than MDA. HNE, for example, reacts with phosphatidylethanolamine and phos-

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phatidylserine (but not with choline) to a fluorophor with maximum excitation and emission at 360/430 nm.[134] A lipid fluorophor with exactly the same spectral characteristics is formed on treatment of microsomes or mitochondria with HNE.[134] Moreover, low density lipoprotein treated with HNE in vitro develops a strong protein fluorescence with an excitation and emission maximum at 360/430 nm, which again agrees exactly with the fluorophor found in oxidized LDL.[331,332] Beppu et al.[328] demonstrated that hexanal, 2-heptenal, 2,4-decadienal, and 2,4,7-decatrienal modify proteins and lipids of human erythrocyte ghosts to fluorescent products with spectral properties identical to those of ghosts treated with linoleic acid hydroperoxide, but different from MDA-treated ghosts. It was also shown by SDS that the proteins were cross-linked, which proves that monofunctional aldehydes have the capacity for cross-linking.

Fluorescent compounds were also reported to be formed on incubation of DNA with MDA.[333] Reevaluation of these experiments[250] with pure MDA preparations, however, gave products with low fluroescence. Incubation of DNA with mono-hydroperoxides, hydroperoxy-epidioxides, hydroperoxy-bis-epidioxides, hydroperoxy-bis-cycloendoperoxides, and dihydroperoxides of linolenate in the presence of $FeCl_3$ and a reducing agent gave products with fluorescence spectra different from those obtained by reaction of DNA with MDA.[334–337] No correlation was found between fluorescence intensity and MDA formation. The fluorescence intensity of MDA-treated DNA or RNA was weaker. Also in the absence of $FeCl_3$ and ascorbate no fluorescent products were formed. A conjugated diene-system in the monohydroperoxide gives higher fluorescent yields than unconjugated hydroperoxides. Linolenatehydroperoxy-epidioxides, linolenate-dihydroperoxides and linoleate dihydroperoxides were the most active compounds. Also, the nature of the reducing agent influences the fluorescent yield. Higher yields were obtained with ascorbic acid than with cysteine indicating a participation of ascorbic acid in the reaction leading to the fluorescent pigment. Radical scavengers such as α-tocopherol or butylated hydroxyanisole depressed fluorophore formation. Based on these findings the authors concluded that some more reactive compounds than MDA are involved. Decadienal or 2-octenal were suggested to be involved in formation of fluorescent DNA pigments. 2,4-Alkadienals and 2,4,7-decatrienal were found to react with DNA to fluorescent products. Reaction of these secondary LPO products may occur after adenine moieties in DNA become accessible by strand-breaking by radicals derived from linoeate-hydroperoxides.

IMMUNOLOGICAL PROPERTIES OF ALDEHYDE MODIFIED PROTEINS

Injection of MDA-treated egg-white lysozyme intradermically into rabbits gave rise to the formation of antibodies.[338–341] The antibody response against MDA-modified lysozyme was different from that developed against native lysozyme, and it was assumed that the antibodies specifically recognized MDA-cross-linked lysine residues with the amino-imino-propen structure.

The recently developed hypothesis that oxidation of LDL and modification of the apolipoprotein B by aldehydic fatty acid degradation products plays a crucial role in foam cell formation and in the pathogenesis of atherosclerosis (for review see 32, 381) has prompted some remarkable studies related to antibodies against aldehyde-modified apolipoprotein B, the protein part of the low density lipoprotein. Haberland et al.[85] were the first to develop a monoclonal antibody against MDA-modified apolipoprotein B. It was again assumed by these authors, although without direct evidence, that the antibody is specific for MDA-modified lysine residues of the apoB. By immunocytochemical methods it was demonstrated that proteins with MDA-modified lysine residues are present in atherosclerotic lesions of rabbit aortas. These investigations were later confirmed and extended by J. Witztum's group[33,84,86] which developed antisera and several types of monoclonal antibodies against LDL modified in vitro by treatment with MDA or HNE. These antibodies appear to be specific for MDA-lysine or HNE-lysine conjugates in proteins, and it was shown that such conjugates occur in oxidatively modified LDL and in atherosclerotic lesions. It has also been reported that human and rabbit sera contain auto-antibodies against MDA- or HNE-modified proteins (for review see 32). Furthermore, it has been shown in cell culture experiments that MDA-modified LDL is recognized and taken up in an unregulated manner by the scavenger receptor present on the cell membrane of monocyte-macrophages. The apoB of the internalized LDL is then degraded to amino acids, whereas the lipid portion (mainly cholesterol and cholesterol-esters) is stored intracellularly in the form of lipid droplets. On prolonged exposure to MDA-modified LDL the macrophages develop into cells with the typical appearance of foam cells (for review see 32). The macrophage scavenger receptor has a rather broad specificity and recognizes many other types of chemically-modified LDL and other proteins. The scavenger receptor complex is probably a family of several receptors differing in affinity to modified proteins. Bovine

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serum albumin (BSA) modified by formaldehyde or MDA (25 mM) as well as by other aliphatic aldehydes is also taken up and endocytosed by mouse peritoneal macrophages and nonparenchymal liver cells via a scavenger receptor mechanism.[244] The receptor recognizing formaldehyde-treated albumin also binds albumin modified by glycolaldehyde, glyceraldehyde, or propionaldehyde, yet not albumin treated with aromatic aldehydes. It was suggested by Horiuchi et al.[383, 384] that this receptor plays a physiological role for the binding and elimination of aldehyde-modified proteins. Competition studies suggest the presence of one receptor recognizing both MDA-BSA and formaldehyde-modified BSA, and another having an affinity towards MDA-BSA, but not towards formaldehyde-modified BSA.[342] MDA-BSA modified with only 10 mM MDA did not affect the binding of highly modified (25 mM) MDA-BSA.[244] Some evidence exists that in MDA-modified BSA the amino-propenal configuration is recognized by the receptor, whereby the double bond is not essential since treatment of MDA-BSA with cyanoborohydride did not affect its ligand activity. Additional cross-linking with glycine, however, markedly diminished the ligand activity of MDA-BSA. Erythrocytes treated with 1 mM MDA are readily phagocytosed by human macrophages.[343] This effect is highly specific for modifications produced by MDA since equimolar amounts of other aldehydes (formaldehyde, acetaldehyde, butyraldehyde, valeraldehyde, glutaraldehyde, acrolein, crotonaldehyde) were without any effect.

With antibodies specific for MDA- or HNE-lysine protein adducts it was also shown by immunocytochemical methods that such aldehyde-modified proteins occur in cultured human fibroblasts after exposure to ascorbic acid.[382] This kind of treatment induced lipid peroxidation and stimulated collagen expression. Immunological studies with aldehyde-modified proteins suggest that the autoantibodies directed against them and the macrophage scavenger receptor are part of the self-defense system removing proteins damaged by aldehydes generated during in vivo lipid peroxiation.

GENOTOXICITY AND MUTAGENICITY

Many genotoxic substances, including carcinogens, are metabolically activated through free radical reactions and can induce lipid peroxidation (for review see 344–348). The possibility therefore exists that endogenously formed aldehydic lipid peroxidation products such as MDA, HNE, or others react with DNA bases and induce mutations. Furthermore, lipid peroxidation and products formed in this process could inhibit certain DNA repair systems[97,306,310] and thus indirectly increase the rate of spontaneous mutation. The pros and contras for the hypothesis of "membrane mediated damage to DNA" as well as the possible involvement of aldehydes were reviewed in several articles recently.[318,347,348] Moreover a comprehensive discussion on the current knowledge of genotoxic effects of HNE will soon appear.[349] We therefore confine ourselves here to a survey in tabular form (Table 8).

As discussed in the previous chapters, MDA, HNE and other α,β-unsaturated aldehydes such as acrolein or crotonaldehyde do react in vitro with nucleosides (Fig. 8, 27, 28, 29, 32). The most reactive base appears to be guanine, which gives with MDA the 1:1 adduct *45* and the 1:2 adduct *46* (Fig. 27), while acrolein and crotonaldehyde give the 1:1 adducts *56*, *57*, *58* (Fig. 32). HNE can give two different 1:1 adducts *26*, *27* (Fig. 8) depending on the reaction conditions. MDA also reacts with deoxyadenosine (Fig. 28) and deoxycytidine. Some recent studies suggest that the oligomeric MDA adducts always result from addition of di- or trimeric MDA molecules present in concentrated MDA solutions (Fig. 14). It is unlikely that di- or trimeric MDA occurs in vivo,[379] and such oligomeric DNA adducts can probably not be formed. In isolated DNA treated with MDA, acrolein or crotonaldehyde the expected 1:1 adduct with the guanine base (formula *45* in Fig. 27 and *57* and *58* in Fig. 32) were found. No clear evidence exists that MDA can cross-link DNA as proposed in Fig. 30. The reaction of HNE with isolated DNA has so far not been investigated in detail and it is also not known if HNE-DNA adducts can be formed in vivo. On the other hand, the in vivo detection of crotonaldehyde and acrolein modified DNA was recently reported by Chung's group.[395–398] At first, an immunoassay based on monoclonal antibodies recognizing the crotonaldehyde-deoxyguanosine adduct *58* was developed.[395] This antibody cross-reacts with the acrolein-deoxyguanosine adduct *57*. Application of this assay to *Salmonella typhimurium* treated with acrolein showed that at the highest acrolein concentration tested (13 mM) about 1 amongst 200,000 deoxyguanosine residues (5 μmol adduct/mol deoxyguanosine) was converted to the exocyclic 1,ON^2-propanodeoxyguanosine with the structure *57*. In the tester strain *S. typhimurium* T100 a correlation existed between number of acrolein-deoxyguanosine adducts and frequency of acrolein-induced mutations. More sensitive and likely more specific for acrolein modified DNA bases is the recently developed ^{32}P-postlabeling method.[396] In case of mice treated topically with crotonaldehyde (5 times over 3 weeks, total dose 1.4 mmol) the ^{32}P-postlabeling method revealed that the skin DNA of the croton-

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Table 8. Genotoxic Effects of MDA, Hydroxyalkenals, Acrolein, Crotonal and Oxidized Lipids.

Authors	Observed Effects
A. *Cell free systems, isolated DNA, nucleosides*	
Fisher et al., 1954 (350)	ox 18:3 but not ox 18:2 decrease viscosity of DNA
Brooks et al., 1968 (333)	DNA incubated with MDA has altered physico-chemical properties and adenine and guanine cross-links
Reiss et al., 1972 (357)	DNA incubated with MDA shows fluorescence at 460 nm
Reiss et al., 1973 (358)	DNA incubated with ox 20:4 shows fluorescence at 420 nm
Borg et al., 1983 (351)	DNA incubated with ox 18:3 gives an ESR signal
Morita et al., 1983 (354)	linoleic acid hydroperoxide produces DSB in the phage PhX 174, DSB enhanced by Fe, Cu
Seto et al., 1983 (245)	MDA produces fluorescent DNA with pyrimido/purinone nucleosides
Chung et al., 1983/1984 (303,304)	crotonaldehyde, acrolein form with guanosine cyclic adducts
Basu et al. 1984 (359)	MDA produces cross-links in complementary DNA
Inouye, 1984 (353)	linoleic acid hydroperoxide produces DSB in the plasmid pBR 322
Nakayama et al., 1984 (352)	DNA incubated with ox 18:3 gives an ESR signal at guanine bases
Nair et al., 1984/1986 (246,249)	MDA produces a 1:1 and 1:3 adduct with adenosine and a 1:3 adduct with cytidine
Ueda et al., 1985 (355)	the DSB produced in plasmid pBR 322 by ox 18:2 correlate with peroxide value
Akasaka et al., 1986 (356)	ox liposomes inactivate the transforming activity of the plasmid pBR 322 the inactivating factor is extractible with chloroform
Marnett et al., 1986 (247)	MDA forms with guanosine a cyclic adduct
Winter et al., 1986 (81)	HNE and HHE form with guanosine cyclic adducts
Basu et al., 1988 (248)	MDA, crotonaldehyde, acrolein form with guanosine cyclic adducts
Sodum et al., 1988 (61,82)	HNE forms in the presence of peroxides with guanosine a 1:1 adduct of the ethenodeoxyguanosine structure
Stone et al., 1990 (400)	adducts formed by reaction of MDA with adenosine
B. *Effects on prokaryotes*	
Mukai et al., 1976 (360)	MDA is mutagenic in *S. typhimurium*
Shamberger et al., 1979 (361)	MDA increases mutation frequency in *S. typhymurium*
Yonei et al., 1981 (362)	MDA induces mutation in *E. coli*
Lutz et al., 1982 (363)	acrolein and crotonal are mutagenic in *S. typhimurium* with 90 min preincubation
Basu et al., 1983 (168)	enzymatically prepared MDA is a weak mutagen in *S. typhimurium*
Akasaka et al., 1985 (365)	*E. coli* treated with peroxidized rat liver microsomes show increased mutation frequency, the increase correlated with MDA
Marnett et al., 1985 (130)	formaldehyde, 2-alkenals, 2,4-alkadienals, 4-hydroxyalkenals, dicarbonyls are mutagenic in *S. typhimurium*
Khudeley et al., 1987 (364)	acrolein is mutagenic in *S. typhimurium*
Foltes et al., 1989 (395)	propanodeoxyguanosine is present in DNA of *S. typhimurium*-treated mice with acrolein
C. *Effects on eukaryotes*	
Yau et al., 1979 (367)	MDA increases mutation in lymphoma cells
Au et al., 1980 (366)	acrolein is cytotoxic and genotoxic to Chinese hamster ovary cells
Bird et al., 1980 (368)	MDA (0.1 mM for 96 h) produces various DNA damages in fibroblasts
Brambilla et al., 1986 (99)	HNE and other hydroxyalkenals produce fragmentation and SCE in Chinese hamster ovary cells
Cajelli et al., 1987 (115)	HNE is a mutagen in V79 Chinese hamster lung cells
Grafström et al., 1988 (305)	acrolein induces genotoxic effects in bronchial epithelial cells
Curren et al., 1988 (306)	acrolein is mutagenic (0.2-0.8 μM) in human Xeroderma pigmentosum fibroblasts, but not in normal fibroblasts
Eckl et al., 1989 (108)	HNE induces genotoxic effects in hepatocytes at subcytotoxic concentrations
Esterbauer et al., 1989 (110)	HNE induces in cultured hepatocytes DNA damages at concentrations being expected to occur in vivo (0.1–1 μM)
D. *In vivo effects*	
Shamberger et al., 1974 (370)	2-stage application of MDA and croton oil increases tumor frequency in Swiss mice
Ferron et al., 1977 (369)	acrolein (4 ppm for 1 year) not carcinogenic in Syrian hamster
Tinsley et al., 1981 (371)	a diet with oxidized polyunsaturated fatty acid increases mammary tumors in mice
Fisher et al., 1983 (372)	MDA and croton oil do not increase tumor frequency in Chinese hamster V 79
Siu et al., 1983 (373)	MDA fed 90 days to mice increases tumor frequency
Bull et al., 1984 (374)	hydroperoxides of linoleic acid and arachidonic acid stimulate in rats colon ornithine decarboxylase
Chung et al., 1986 (375)	crotonal (0.6 or 6.0 mM in drinking water, 113 weeks) increases frequency of liver tumors in rats
Lijinsky, 1987/1988 (376,377)	acrolein, oral to rats is of low carcinogenicity
Spalding, 1989 (378)	MDA (sodium salt) is carcinogenic in mice and rats
Chung et al., 1989 (396)	cyclic 1,ON^2-propanodeoxyguanosine adducts in DNA of mice treated with crotonaldehyde and rats treated with *N*-nitrosopyrrolidine

HNE = 4-hydroxynonenal, HHE = 4-hydroxyhexenal, 18:2 = linoleic acid, 18:3 = linolenic acid, ox = oxidized, DSB = double strand breaks, SCE = sister chromatid exchange.

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aldehyde-treated animals contained about 0.24 μmol crotonaldehyde-deoxyguanosine adduct *58* per mol deoxyguanosine, that is, one out of 4 million guanine bases was modified by crotonaldehyde. The ^{32}P-postlabeling method also allowed to assess the possible formation of crotonaldehyde-deoxyguanosine adducts in rats treated with *N*-nitrosopyrrolidine.[396,397] This hepatocarcinogenic nitrosamine is metabolized in the liver in part to crotonaldehyde. Rats, which received *N*-nitrosopyrrolidine in the drinking water (total dose 1.0 mmol) contained in the liver DNA the crotonaldehyde modified deoxyguanosine (formula *58*, Fig. 32), although the concentration was rather low, that is, 0.056 μmol/mol deoxyguanosine, which corresponds to 1 residue amongst 16 million. Nevertheless these are the first reports showing the in vivo formation of adducts between α,β unsaturated aldehydes and DNA. An attempt was also made, to demonstrate the in vivo formation of 4-hydroxyalkenal DNA adducts, for that tritium labeled 4-hydroxyhexenal was injected into the hepatic portal vein of rats.[147] No detectable radioactivity was associated with the liver DNA. Based on the specific radioactivity in can be calculated that the number of modified bases, if they existed at all, must have been less than 1:50000. The in vitro reaction of DNA bases or DNA itself with aldehydes requires rather high concentrations (mM and more) and long reaction times, which indicates that in vivo modification of DNA by MDA, 4-hydroxyalkenals, or other α,β unsaturated aldehydes will be a rare event. In agreement with that are the findings by Chung et al.[396,397] according to which the levels of such adducts found in vivo are very low. Nevertheless sufficient evidence exists that aldehydes can produce a number of genotoxic effects including mutations and induction of tumors (Table 8).

MDA is mutagenic to bacterial and mammalian cells, and carcinogenic in rodents. Acrolein and, to a lesser extent, crotonaldehyde are mutagenic in bacteria. Acrolein can also, as recently shown, induce mutations in mammalian cells. 4-hydroxyalkenals, too, are mutagenous in the *Salmonella typhimurium* assay, and mutagenicity was also shown in V79 Chinese hamster lung cells. Furthermore it was shown that HNE and other 4-hydroxyalkenals induce genotoxic effects in various mammalian cells. A particularly sensitive test system for genotoxicity of aldehydes are primary cultures of rat hepatocytes. With this type of cells it was shown[108,110] that concentrations of 10, 1, or 0.1 μM HNE are genotoxic and lead to a significant increase of micronuclei, sister chromatid exchange and chromosomal aberrations. More or less the same activity was shown by 2-nonenal, whereras the saturated aldehyde nonanal was not genotoxic up to 100 μM, the maximal dose tested. Taking into account that the endogenous concentration of HNE in liver is in the range of about 0.1-0.5 μM (Table 2), one might assume that HNE or similar aldehydes generated by basal lipid peroxidation are a constant source of DNA damage. If that is so, it will be critical to know if such a damage is irreversible, mutagenic, or even carcinogenic.

CONCLUSION

Food chemists have long known that autoxidation of fats and oils, in particular those rich in PUFAS is accompanied by the formation of aldehydes.[4-6] It is therefore not surprising that aldehydes are also formed, when biological membranes containing polyunsaturated lipids undergo lipid peroxidation.[2-4] The spectrum of aldehydes which can be formed in true biological samples however is much more complex than in simple fats and oils and the concentration of aldehydes is usually very low. That and other factors (e.g., complexity of biological sample, difficulties to discriminate between free and bound aldehydes) makes the analysis of aldehydes in tissues, cells and subcellular fractions rather difficult. This explains that successful analysis based on HPLC or GC-MS were only developed in the last decade (Table 1, 2). Although considerable progress has been made by several laboratories one must admit that these analyses are far beyond routine assays as they for example exist for eicosanoid analysis. Moreover, it seems also important to remember, that β-scission of a hydroperoxy fatty acid — which is likely the major reaction by which aldehydes are formed during lipid peroxidation — results in two different classes of aldehydes. One, which are fragments derived from the methyl-terminus of the fatty acid chain and another one where the aldehyde function remains at the parent lipid molecule.[4,7] All analysis has so far been confined to the former class, which includes *n*-alkanals, 2-alkenals, 2,4-alkadienals, 4-hydroxyalkenals and malonaldehyde. The pattern of these aldehydes solely depends on the PUFAS yet not on the nature of the parent lipid molecule in which they are bound. Thus all lipids containing omega-6 PUFAS (18:2, 20:4) will give rise to 4-hydroxynonenal and hexanal, whereas omega-3 PUFAS will form 4-hydroxyhexenal and propanal. Similarly malonaldehyde will be formed from lipids containing PUFAS with 3 or more methylene interrupted double bonds (mainly 20:4 and 22:6). The lipid-aldehydes, that is, the counterparts of the medium and short chain aldehyde fragments, should also exist in peroxidized biological samples, however no methods are presently available which would allow to separate and identify them. We have repeatedly emphasized[3,4,7,9] that this would be important since some of these lipid-aldehydes might have comparable if not higher biological activities than the medium and short chain counterparts.

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Research in the recent years has mainly focused on some typical representative aldehydes such as 4-hydroxynonenal, 4-hydroxyhexenal, and MDA. One reason for that, among others, is that these aldehydes can be prepared by chemical synthesis in quantities sufficient for biological testing. Other aldehydic lipid peroxidation products which might have interesting biological properties, as, for example, 4,5-dihydroxydecenal or 4-hydroperoxynonenal were hardly studied since their preparation even in small quantities is difficult.

That free HNE occurs in various peroxidized biological samples has been demonstrated independently by many groups with different methods (Tables 1,2) and it can therefore be taken as granted. Some uncertainty still exists on the basal, physiological level of HNE as for example in rat liver or human plasma. A steady-state concentration of 2.8 nmol HNE/g liver as reported by one group (Table 2) appears much too high in view of the high HNE-metabolizing capacity of the liver. We would assume that the HNE concentration in liver and also in normal human plasma is rather close to the detection limit (0.1 to 0.3 nmol/g tissue or plasma) of the presently available methods. More work should be done to improve this analysis and to ascertain the physiological value of HNE and possible correlations between increased values and diseases.

The hypothesis, that HNE and other reactive aldehydes produced endogenously during lipid peroxidation act as "second toxic messengers" for free radical events is based on the assumption that aldehydes interact with essential cell components (e.g., membrane proteins) and thereby cause disturbance of cell functions (Fig. 1). In fact, HNE administered to animals, tissues, or cells produces a great diversity of deleterious effects (Tables 4, 5). But this by its own does of course not prove that HNE produced intracellularly does the same. Until recently a major argument against a pathological role of HNE (the same is true for MDA) in lipid peroxidation was that the in vivo concentrations can not reach levels sufficient to effect enzyme activities and modify proteins and/or DNA. A number of newer findings however, indicate that even very low HNE concentrations (0.1–1 μM), which can likely be reached in vivo in certain tissues exposed to oxidative stress (Table 2), can produce some remarkable effects: Stimulation of phospholipase C and adenylate cyclase, recruitment of neutrophils, reduction of c-myc expression, induction of genotoxic effects (see Table 4). That such HNE-mediated effects may have a pathological and possibly also a physiological significance in vivo is therefore rather likely, yet a definite proof would require many more investigations (for review see also ref. 399). The same that has been said about HNE holds also for MDA or other aldehydic lipid peroxidation products. Here too, it is presently not possible to make conclusions which one of the many in vitro effects have an importance in vivo.

A significant development which could in the future help to bridge this gap, is the application of antisera and monoclonal antibodies against HNE and MDA modified proteins.[32,38,84–86] Using such antibodies it was recently shown with immunostaining that HNE- and MDA-conjugated proteins are present in atherosclerotic lesions of rabbits and that autoantibodies against such aldehyde-modified proteins occur in human plasma. This is the first clear evidence that proteins modified by aldehydic lipid peroxidation products do in fact occur in vivo and probably play a role in the pathogenesis of a human disease (i.e., atherosclerosis). Antibodies were also successfully used for immunocytochemical demonstration of HNE and MDA-modified proteins in fibroblasts in which lipid peroxidation was initiated by ascorbate/iron.[382] These highly sensitive and specific immunological techniques are very promising tools which will allow researchers in the future to study lipid peroxidation and the role of aldehydes at the cellular level. Moreover it should not be too difficult to develop ELISAs for routine quantification of HNE- and MDA-modified proteins in cells, tissue and human plasma. In parallel, the chemical structure of the epitopes on the aldehyde modified proteins recognized by the antibodies should be characterized. We do hope, that this review stimulates interdisciplinary work and contributes to the scientific progress in the field of lipid peroxidation, aldehydes, and free radicals in biology and medicine.

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ABBREVIATIONS

Apo B — apolipoprotein B
EATC — Ehrlich ascites tumor cells
GSH — glutathione
HNE — 4-hydroxynonenal
LDL — low density lipoprotein
MDA — malonaldehyde
PUFA — polyunsaturated fatty acids
TBA — thiobarbituric acid
TBARS — thiobarbituric acid reactive substances
TCA — trichloroacetic acid

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